Nicotinic autoreceptors in the rat hippocampus regulate acetylcholine release

Wilkie, Graham I.

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NICOTINIC AUTORECEPTORS IN THE RAT HIPPOCAMPUS REGULATE ACETYLCHOLINE RELEASE

BY

GRAHAM I. WILKIE

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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Submitted by **Graham Ian Wilkie**

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Signed: 

ABBREVIATIONS

AC	adenylyl cyclase
AD	Alzheimer's disease
ADP	adenosine diphosphate
ATP	adenosine triphosphate
AcCoA	acetyl Coenzyme A
ACh	acetylcholine
AChR	acetylcholine receptor
AChE	acetylcholinesterase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANTX	(+)-anatoxin-a
αBgt	α-Bungarotoxin
BAPTA	1,2-bis(2-Aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BSA	bovine serum albumin
Ca^{2+}	calcium
Ch	choline
ChAT	choline acetyltransferase
CNS	central nervous system
CX	cortex
DHβE	dihydroβerythroidine
DMPP	1,1 dimethyl-4-phenylpiperazinium iodide
DNA	deoxyribose nucleic acid
EDTA	diaminoethane tetra-acetic acid, disodium salt
EGTA	ethyleneglycol-bis-(βaminoethyl ether)-N,N,N',N'-tetraacetic acid
Fx	fractions 1-5, S1 fractionated using a 4-step Percoll gradient
GABA	γ-aminobutyric acid
G protein	guanine nucleotide regulatory protein
[^{3}H]QNB	tritiated quinuclidinylbenzilate
5HT	5-hydroxytryptamine
K^+	potassium

kDa	kilodaltons
HP	hippocampus
[^{125}I]αBgt	iodinated α-bungarotoxin
icv	intracerebroventricular
i.p.	intraperitoneally
IP_3	triphosphotidylinositol
LDH	lactate dehydrogenase
LGIC	ligand gated ion channel
mAb	monoclonal antibody
mAChR	muscarinic acetylcholine receptor
MLA	methyllycaconitine
mRNA	messenger RNA
MSA	medial septal area
Na^+	sodium
nAChR	nicotinic acetylcholine receptor
NADH	nicotinamide adenine dinucleotide
nBgt	neuronal bungarotoxin
nBM	nucleus basalis of Meynert
P2	plasma membrane preparation, pellet from medium speed centrifugation
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEI	polyethyleneimine
PI	phosphotidylinositol
PKC	protein kinase C
PVP	polyvinylpyrolidone
QNB	quinuclidinylbenzilate
RNA	ribose nucleic acid
s.c.	subcutaneous
SDS	sodium dodecyl sulphate
S1	supernatant fraction from low speed centrifugation

SUMMARY

The pathology of Alzheimer's disease highlights the importance of hippocampal and cortical presynaptic cholinergic receptors. The presynaptic hippocampal nAChRs represent one possible avenue for development of rational cholinergic drug therapy. The fact that the administration of nicotine apparently enhanced attention in Alzheimer patients (Sahakian et al., 1989), demonstrates the value of targeting the nicotinic receptors, but unfortunately the side effects of nicotine are legion, precluding any major therapeutic role for the drug. The propensity of the nAChRs to desensitise upon exposure to agonist also means that potential drugs would have a limited concentration 'window' in which to facilitate the release of ACh. Clearly, a more complete pharmacological understanding of these presynaptic nAChRs would assist the rational design of drugs to combat Alzheimer's disease and other dementing diseases.

A reproducible superfusion protocol was established for the Brandell SP-06 apparatus, initially based upon the work of Thorne (1990). Using this apparatus, superfusion of [^{3}H]choline-loaded cortical, then hippocampal synaptosomes, was characterised with respect to the optimal synaptosomal loading concentration, response to a general depolarising agent (KCl), and Ca^{2+}-dependence.

Dose-dependent agonist-evoked [^{3}H]ACh release from hippocampal synaptosomes was demonstrated in response to a nicotine challenge. Both agonist- and KCl-evoked release was shown to be Ca^{2+}-dependent. Nicotine- but not KCl-evoked release, was shown to be mediated via presynaptic nAChRs using the nicotinic antagonist DHβE. In addition to nicotine, a variety of agonists was shown to mediate dose-dependent [^{3}H]ACh release, enabling the estimation of EC_{50} values for the following agonists: nicotine, cytisine, (+)-anatoxin-a, iso-arecolone and ABT-418. A characteristic pattern of nAChR desensitisation at higher agonist concentration, was seen for each agonist. Repetitive agonist stimulation revealed profound nAChR desensitisation, even at agonist concentrations producing sub-maximal transmitter release. The peptide, substance P (SP) did not affect the apparent affinity or binding density of [^{3}H]nicotine. Similarly, nicotine does not alter the binding characteristics of [^{3}H]SP. Agonist-induced nAChR desensitisation was affected by SP in a complex manner, consistent with the possible interaction of hippocampal tachykinin receptors.

In a comparison of control rats, and MSA and nbM lesioned rats (using excitotoxic amino acids), small decreases in ChAT activity were found with no corresponding decrease in [^{3}H]nicotine binding density. Preliminary studies using the technique of *in vivo* microdialysis, confirmed that systemic injection of nicotine was able to evoke ACh release in the hippocampus of freely moving rats.

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PUBLICATIONS RESULTING FROM WORK IN THIS THESIS

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QUAND vous serez bien vieille, au soir à la chandelle,
Assise auprès du feu, devidant et filant,
Direz chantant mes vers, en vous esmerveillant:
Ronsard me celebroit du temps que j'estois belle.

Lors vous n'aurez servante oyant telle nouvelle,
Desja sous le labeur à demy sommeillant,
Qui au bruit de mon nom ne s'aille resveillant,
Benissant vostre nom de louange immortelle.

Je seray sous la terre, et, fantosme sans os,
Par les ombres myrteux je prendray mon repos:
Vous serez au fouyer une vieille accroupie,

Regrettant mon amour et vostre fier desdain.
Vivez, si m'en croyez, n'attendez à demain:
Cueillez dès aujourd'huy les roses de la vie.

P. De Ronsard

For Christina.

WHEN you are old and grey and full of sleep,
And nodding by the fire, take down this book,
And slowly read, and dream of the soft look
Your eyes had once, and of their shadows deep:

How many loved your moments of glad grace,
And loved your beauty with love false or true,
But one man loved the pilgrim soul in you,
And loved the sorrows of your changing face:

And bending down beside the glowing bars,
Murmur, a little sadly, how Love fled
And paced upon the mountains overhead
And hid his face amid a crowd of stars.

W.B. Yeats

1.1. ALZHEIMER'S DISEASE

One of the most feared and devastating aspects of ageing is the deterioration of memory and other mental processes that occurs with increasing frequency in advancing years. About 5% or more of the population above the age of 65 years suffers from dementia, a severe impairment in cognitive functions; an additional 10% of individuals exhibit mild-to-moderate abnormalities in their cognitive abilities (Blessed, 1980). The most common cause of this dementia is Alzheimer's disease (AD-here used to refer to both the senile and presenile Alzheimer's disease). Its characteristic feature of a relentlessly progressive decline in intellectual abilities in the absence of marked changes in alertness or sensory or motor abilities (Cummings & Benson, 1983), the lack of an effective treatment, and its unknown aetiology (Henderson, 1986) have combined to make AD a major health problem. Mental infirmity is the major reason for the confinement of elderly individuals in nursing homes; and, in the USA for example, the cost of nursing home care alone for patients whose chief symptom is dementia was estimated to exceed $6 billion per year by 1980 (Coyle *et al.*, 1983). Today, it is estimated that the cost to American society for diagnosing and managing Alzheimer's disease, primarily for custodial care, is in the region of $80 billion annually (Selkoe, 1991).

Generally the onset of senile dementia is heralded by impairments in recent memory. Affected individuals may be able to recall in considerable detail life events from the distant past, but they cannot remember what occurred just minutes earlier. Inevitably, higher cognitive functions deteriorate and the patients lose the ability to read, write, calculate, or use language appropriately. The loss of cognitive abilities may be accompanied by psychiatric symptoms such as irritability, emotional lability, paranoid delusions, and hallucinations. Affected individuals remain alert until the terminal stages; and the dementia occurs commonly in the absence of focal neurological deficits such as paralysis or sensory loss, which frequently accompany cerebrovascular disease.

Presenile dementia of the Alzheimer's type is a rare disorder in which the individuals, typically in their fifties, develop a progressive deterioration of cognitive functions indistinguishable from senile dementia. The demonstration that the pathological alterations in the brains of more than half of elderly demented individuals are similar to

those found in the brains of patients suffering from the presenile form of AD (reviewed in Coyle *et al.*, 1983) suggests that these are related disease processes.

1.1.1. ALZHEIMER PATHOLOGY

Alzheimer's disease was first described by Alois Alzheimer in 1907 (Alzheimer, 1907). At post-mortem, he wrote: "Scattered through the entire cortex, especially in the upper layers, one found miliary foci that were caused by the deposition of a peculiar substance in the cerebral cortex." Evidence has emerged in the past decade that this peculiar substance is a protein fragment - the β-amyloid peptide (discussed below, section **1.1.1.1.**). In 1911 Simchowicz (Simchowicz, 1911) drew attention to the common finding of granovascular degeneration, that is, intracellular vacuoles, in hippocampal pyramidal neurons, and more recently cerebrovascular amyloidosis and the presence of Hirano bodies have been recognised as common pathological findings (Glenner, 1983).

As alluded to previously, the pathology is complex and consists of granovascular degeneration and the presence of senile plaques and neurofibrillary tangles (NFTs). Silver staining techniques have shown the senile plaques to be 20-200 nm in diameter (Marchbanks, 1982) which consist of abnormal axon terminals (derived from cholinergic, adrenergic and somatitinergic neurones; Perry & Perry, (1985) and dendritic processes, often intermingled with astrocytes and microglial cells, associated with a core of extracellular amyloid (Perry *et al.*, 1978; Marchbanks, 1982; Coyle *et al.*, 1983). The amyloid peptide subunit (A4 or β protein) encoded on chromosome 21, is an insoluble, highly aggregating, small polypeptide of 4.5 kilodaltons (kDa; Kang *et al.*, 1987) which has a high co-localisation with aluminosilicates (Candy *et al.*, 1986). This has lead to the hypothesis that aluminium may be an initiating factor in the disease. However it may be the amyloid peptide that traps the aluminium and so elevated aluminium is probably secondary in the progression of the disease (McGeer, 1984).

The NFTs, particularly in the perikaryon of pyramidal cells, are composed of bundles of tightly packed paired helical filaments (PHFs; 10-12 nm in diameter; Marchbanks, 1982; Coyle *et al.*, 1983), consisting largely of the microtubule-associated protein, tau (section **1.1.1.2.**) in an abnormal state of phosphorylation (Mandelkow & Mandelkow, 1993). There have been opposing reports on whether the correlation between the severity of dementia (and the reduction in ChAT) is better mapped to the number of senile plaques (Perry *et al.*, 1978; Sitaram, 1984) or NFTs (Wilcock & Esiri, 1982;

Wilcock *et al.*, 1982). However there is a high density of both these pathological features in the brain of patients with Alzheimer's disease.

1.1.1.1. Amyloid peptide and Alzheimer's disease

Senile plaques are extracellular deposits of a heterogeneous substance called 'amyloid'. They also contain dystrophic neurites (neural cell processes) and glial cells. Although plaques are particularly abundant in the cerebral cortical parenchyma of AD victims, other extracellular amyloid deposits occur in the walls of cerebral and meningeal blood vessels.

Glenner & Wong (1984) isolated and partially sequenced the major protein constituent of cerebrovascular amyloid deposits, a 4 kDa peptide. Subsequently, Masters *et al.*, (1985) showed that this 39-43 amino acid molecule called the β-amyloid peptide or peptide, was also the principle constituent of senile plaques. Other proteins, including ubiquitin, apolipoprotein E (apoE; discussed in section **1.1.1.3.**), interleukin-1, lysosomal hydrolases and α_1-antichymotrypsin, are also found in plaques.

When the first full-length cDNA encoding the β-amyloid peptide was cloned and sequenced (Kang *et al.*, 1987) it was found to encode a protein that is much larger than the 4 kDa β-peptide. Hence, the β-peptide is derived by proteolysis from this larger protein, called the amyloid precursor protein (APP). APP is a large, multidomain protein containing a single membrane-spanning region, with the amino terminus extending into the extracellular environment, and a short region at the carboxyl terminus projecting into the cytoplasm. Approximately two thirds of the β-amyloid peptide portion of APP is extracellular, whereas the remaining third is integrated into the membrane.

Expression of APP is complex. The primary RNA transcript of APP undergoes differential splicing to generate mRNAs coding for at least five forms of APP. APP is also post-translationally modified: the molecule undergoes O- and N-glycosylation, phosphorylation, and sulphation on tyrosine residues (Ashall & Goate, 1994). APP is also regulated compartmentally within the cell: surface APP can be secreted or internalised into the endosomal-lysosomal system, to add to this complexity, APP is also processed by multiple proteolytic pathways.

What has emerged from investigations of the rare, familial forms of AD is that mutations that lead to AD can occur in different genes in different families, and that different mutations can occur at the same genetic locus in different families. The gene encoding APP was mapped to chromosome 21 (St. George-Hyslop *et al.*, 1987), and this suggested that the AD-like pathology seen in Down's syndrome was due to overexpression of the APP gene. This idea was further supported by the discovery that some familial forms of AD were linked to chromosome 21.

Levy *et al.*, (1990) showed that patients with Dutch-type amyloidosis (HCHWA-D) have a point mutation within the β-peptide sequence of APP at amino acid position 22, causing a glutamate residue to be substituted for a glutamine residue (Levy, *et al.*, 1990) patients with HCHWA-D do not develop AD, but they do have diffuse β-amyloid deposits within the cerebral cortex, and cerebrovascular β-amyloid deposits, these patients are prone to brain haemorrhage. This discovery was the first to show that a specific defect in the APP gene can cause β-amyloid to be deposit.

Persuasive evidence that APP can have a primary role in AD came from the discovery by Goate *et al.*, (1991) that a point mutation in the APP gene (position 717, near the carboxy-terminal amino acid of the β-peptide) co-segregated with the disease in at least one early-onset AD family. Subsequently, APP mutations at the same and other codons within the APP gene were found in other AD families. None of these mutations occurs within the β-amyloid peptide of the APP molecule, but they do occur very close to the ends of the β-peptide sequence.

Point mutations in the APP gene are not always the cause of familial AD. Recent research has shown that a mutation in a gene on chromosome 14 can also cause familial AD (Ashall & Goate, 1994). Chromosome 14 mutation(s) accounts for the majority of cases of early-onset AD. The identity of the genes (or genes) involved is not yet clear, and whether or not it encodes a product that is directly involved in APP metabolism has yet to be determined. A gene on chromosome 19 also appears to be linked to some forms of late-onset AD. This may be the apoE gene (section **1.1.1.3.**).

The conclusion that familial AD is genetically heterogenous and can apparently be caused by many different genetic defects on different chromosomes should perhaps come as no surprise. AD is very common, occurs in many ethnic groups and is similar in pathology to the normal process of brain ageing. It is reasonable that the syndrome that is known as

AD could arise in somewhat different forms from many distinct genetic alterations. All these alterations, however, appear to act through a critical common mechanism involving the increased deposition of β-amyloid peptide.

1.1.1.2. Tau protein and Alzheimer's disease

As outlined above, NFTs and their constituents, the PHFs, consist largely of the microtubule-associated protein tau in an abnormal state of phosphorylation. Normal tau protein is abundant in axons, where it stabilises microtubules. It consists of several isoforms generated by alternative splicing of a single gene. The carboxy-terminal half contains three or four imperfect repeats which contribute to microtubule binding and are homologous to other microtubule-associated proteins (MAPs). Abnormal tau is more extensively phosphorylated than normal tau, aggregates irreversibly into PHFs and is highly resistant to proteases. It can be differentiated from its normal counterpart by SDS gel electrophoresis, where the abnormal protein runs at 60-68 kDa, compared with 47-67 kDa for the normal protein.

Phosphorylation of normal tau generally occurs at Ser-Pro (SP) or Thr-Pro (TP) motifs (tau has seven SP and ten TP motifs), suggesting that the kinase responsible belongs to the class of proline-directed kinases such as the 42 kDa MAP kinases. The different Alzheimer-like characteristics of pathological tau might depend on different causes affecting different sites, and with different consequences. For example many authors have reported that phosphorylation of tau or other MAPs affects microtubule assembly (Mandelkow & Mandelkow, 1993), illustrating that such properties as microtubule binding, assembly, and dynamic instability might be differently affected by tau phosphorylation.

1.1.1.3. ApoE and Alzheimer's disease

ApoE is associated with NFTs and β-amyloid peptide in senile plaques. It also appears to play a part in the redistribution of lipids that follows deafferentation and neurodegeneration in the brain. The gene for apoE is on chromosome 19, within the genomic region previously associated with late-onset familial AD (Poirier *et al.*, 1993).

ApoE is a polymorphic protein associated with plasma lipoproteins. It interacts with the 'remnant receptor' (apoE receptor) and the low-density-lipoprotein (LDL) receptor (apoE/B receptor) of the liver and other organs to modulate the catabolism of triglyceride

rich lipoprotein particles. ApoE is unique among apolipoproteins in that it has a special relevance to nervous tissue: it is involved in the mobilisation and redistribution of cholesterol in repair, growth, and maintenance of myelin and neuronal membranes during development or after injury (Poirier *et al.*, 1993).

The importance of apoE is underscored in AD by its presence within the plaques and dystrophic neurites that characterise AD (Namba, *et al.*, 1991), by the fact that apoE mRNA is reduced in the hippocampus in AD (Poirier, *et al.*, 1991) and by the ability of apoE to bind tightly to the soluble and insoluble forms of β-amyloid (Namba *et al.*, 1991).

ApoE is encoded by a gene on the long arm of chromosome 19, within a region previously associated with familial late-onset AD (Poirier *et al.*, 1993). Common apoE polymorphisms are determined by alleles designated ε4, ε3, and ε2 (Mahley, 1988), and the finding that the apoE allele ε4, confers a high probability that an individual will develop late-onset AD, was reported recently (Saunders *et al.*, 1993). A quarter of the population of the world carries this allele, and 2-3% of people are homozygotes. Penetrance of AD in homozygotes is believed to exceed 90% and heterozygotes are at substantially increased risk.

The co-localisation of apoE with the major neuropathological features of AD suggests some relationship to the cause of AD. One possibility is that the age-related decline in cell number and lipid content that happens normally in the human brain is exacerbated by the presence of certain apoE alleles in susceptible individuals. Furthermore if apoE disrupts lipase activity (Yamada & Murase, 1980) in the brain and alters the transport of cholesterol and phospholipids in brain areas vulnerable to ageing, a direct consequence would be aberrant and/or defective reinnervation and poor synaptic plasticity.

1.1.2. CHOLINERGIC RECEPTOR CHANGES

Since neurotransmitter-specific neuronal systems have been shown to have a role in the pathophysiology of disorder like Parkinson's disease and Huntington's disease, investigators have begun to focus on the role of neurotransmitters in the symptoms of disorders of cognition and memory.

AD is characterised by an extensive degeneration of the cholinergic system in the human nucleus basalis of Meynert, (nBM) neocortex and hippocampus (Hardy *et al.*, 1985;

Candy *et al.*, 1986; Quirion *et al.*, 1986; Plotkin *et al.*, 1986; Araujo *et al.*, 1988), mainly based upon measures of choline acetyltransferase activity (ChAT). The reduction in neuronal input is due to atrophy in the cholinergic nuclei: the nBM has a severe reduction in ChAT of up to 75% (Whitehouse *et al.*, 1981, 1982; Sitaram, 1984); the medial septal nucleus and the nucleus of the diagonal band have shown 55% and 65% reductions in ChAT activity, respectively (Henke & Lang, 1983). The validity of using ChAT as a marker for Alzheimer's disease was shown by Perry *et al.*, (1978) who quantitatively correlated the reduction in cortical and hippocampal ChAT with both the numbers of neuritic plaques and the severity of dementia as measured by cognitive and behavioural rating scales.

The bulk of the neurochemical work on Alzheimer's disease has concentrated on measuring transmitter or metabolite levels and/or enzyme activities for synthesis or metabolism. Using radiolabelled transmitters, agonists or antagonists, changes in receptor densities could also be determined (Davies & Verth, 1978). The decrease in ChAT activity in post-mortem AD brains also correlated with a decrease in cortical and hippocampal nicotinic receptor density, and muscarinic M2-subtype receptor density (Perry *et al.*, 1986; Lapchak *et al.*, 1989). Changes in the density of M1-subtype muscarinic receptors, as defined by high affinity [^{3}H]pirenzipine binding, do not correlate with changes in ChAT activity however (Lapchak *et al.*, 1989). The existence of presynaptic nAChRs was established in a number of studies (Wonnacott *et al.*, 1989; Wonnacott, 1990; Lapchak *et al.*, 1989; Boksa, 1987; Rowell *et al.*, 1984), and are thought to function as positive feedback autoreceptors. The fact that decreases in the M2 mAChR population mirror the changes in nAChR density, strengthen the argument that M2 mAChR are also presynaptically distributed with the nAChR at least in the cortical and hippocampal regions studied, functioning as negative feedback autoreceptors. The degeneration of cholinergic axons in Alzheimer's disease would spare the majority of postsynaptically located M1 mAChR, hence no significant changes in receptor density are observed.

1.2. THE HIPPOCAMPUS

1.2.1. CHOLINERGIC INNERVATION

Although ACh was the first substance to be established as a transmitter, both in the PNS where it was retrospectively identified as the chemical substance responsible for the reported vagal inhibition of the heart, and the CNS (motor axon collateral excitation of

Renshaw cells), the effects of synaptically released ACh in mammalian brain have been difficult to demonstrate. Numerous biochemical and histochemical studies over the years have described the widespread distribution of ACh and its metabolic enzymes AChE and ChAT in the brain, and iontophoretic studies have delineated the distribution of cholinoceptive cells. Whilst the cholinergic innervation of most brain structures is diffuse and in most cases relatively sparse compared to other inputs (Nicoll *et al.*, 1985), one exception to this general rule is the septo-hippocampal pathway originating primarily from neurons in the medial septal nucleus and from neurons in the diagonal band. The pioneer studies of Lewis and Shute (1967), established that this pathway is particularly rich in AChE and the loss of both AChE and ChAT in the hippocampus following lesions to this pathway led these authors to conclude that the septo-hippocampal pathway is the major source for the cholinergic input to the hippocampus. More recent immunohistochemical studies on the distribution of ChAT by Houser *et al.*, (1983) and Cuello & Sofroniew, (1984) are in complete accord with the earlier conclusion, although non-cholinergic afferents have clearly been identified in this pathway (Wainer *et al.*, 1985).

1.2.2. CHOLINERGIC RECEPTORS IN THE HIPPOCAMPUS

Both nicotinic and muscarinic AChRs coexist in the hippocampus. Although muscarinic antagonist binding studies originally indicated a uniform population of muscarinic receptors, but by 1980, it was realised that the mAChR-mediated actions of ACh could not be accounted for by a single receptor subtype. In particular, radioligand-binding studies revealed tissue -specific differences in the affinity of the antiulcer drug pirenzepine, suggesting the presence of at least two receptor subtypes (Hammer *et al.*, 1980). A high-affinity antagonist site, M1, prevalent in the hippocampus, is well characterised and apparently unaltered in Alzheimer's disease (Birdsall *et al.*, 1984), although antagonist sites of medium and low affinities - grouped as M2 - have been reported to be reduced (Caulfield *et al.*, 1982). One possible explanation for this differential reduction in M2 mAChR compared to M1 receptors is a difference in localisation. M1 receptors are thought to be postsynaptic, whereas M2 receptors may be colocalised with presynaptic nicotinic receptors (Perry *et al.*, 1989; section **1.1.2.**).

1.3. ACETYLCHOLINE RECEPTORS

In 1914, Sir Henry Dale provided the basis for the classical definition of muscarinic and nicotinic acetylcholine receptors: muscarinic receptors are selectively activated by

muscarine and blocked by atropine; nicotinic receptors are activated by nicotine and blocked by curare. This definition lasted over 60 years, despite isolated reports that the picture might not be so simple. It is now known, as a result of molecular biological studies, that there are multiple variants of both muscarinic and nicotinic receptors. These receptors are members of two quite distinct gene superfamilies and only share the property of being activated by the same ligand, acetylcholine.

The nAChR and mAChR have opposing effects on ACh release from presynaptic nerve terminals. Nicotine and nicotinic agonists have an excitatory effect on basal ACh release, an effect which is antagonised by nicotinic antagonists (Briggs & Cooper, 1982; Moss & Wonnacott, 1985; Rowell & Winkler, 1984; Wessler et al., 1986; Wonnacott *et al.*, 1989; Nordberg *et al.*, 1989; Thomas *et al.*, 1993; Wilkie *et al.*, 1993). In contrast, muscarine and its agonists have an inhibitory effect on evoked ACh release which can be blocked by muscarinic antagonists (LeFresne *et al.*, 1978; Marchi *et al.*, 1981; 1983; Briggs & Cooper, 1982; Baux *et al.*, 1987). Briggs & Cooper (1982) hypothesised that the nAChR acts as an accelerator of ACh release, at low ACh concentrations, while the mAChR acts as a brake on release, at high ACh concentrations, thereby achieving fine regulation of ACh release. The two types of cholinergic receptor differ both in structure and mechanism of action.

1.3.1. MUSCARINIC RECEPTORS

By sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) the mAChR comprises a single polypeptide of 70-80 kDa (Birdsall *et al.*, 1979; Peralta *et al.*, 1987). Sequence analysis, electron diffraction and limited proteolysis studies have shown the receptor to have a short extracellular glycosylated N-terminal region which is thought to be associated with the binding site (Hall, 1987), see figure **1.1.** There are seven hydrophobic regions of 20-25 amino acids which span the membrane (Birdsall & Hulme, 1987; Hall, 1987; Peralta *et al.*, 1987). Intracellularly there is a long cytoplasmic loop between transmembrane regions 5 and 6 that is thought to be the site of interaction with G-proteins (see below). The cytoplasmic hydrophilic C-terminal sequence contains serine and threonine residues that can be phosphorylated by protein kinases. This phosphorylation may play an important role in desensitisation and internalisation of the receptor (Birdsall & Hulme, 1987; Hall, 1987). The model (figure **1.1**) leaves little of the N-terminal structure for selective and stereospecific ligand binding so the binding site may lie in the pocket formed by the transmembrane domains (Kerlavage *et al.*, 1987).

1.3.1.1. Mechanism Of Action Of mAChRs

The muscarinic response of the mAChR is slow (latency 100 ms and duration 300-500 ms duration; Sokolovsky & Bartfai, 1981) due to its integrated response via three proteins, see figure **1.2.** The binding of ACh initiates the binding of a guanine nucleotide regulatory protein (G protein) to the mAChR, which acts as a transducer to an appropriate effector system (Bourne, 1986; Hall, 1987). G proteins are a family of heterotrimeric proteins: a common β/γ complex ($G_{\beta\text{-}\gamma}$; 35 kDa and 10 kDa, respectively) that is thought to anchor the protein to the cytoplasmic face of the membrane and an α chain (G_{α}; 41-45 kDa) that binds GTP and determines the specificity of the G protein for receptor and effector (Gilman, 1984; Bourne, 1986). More recent evidence has also suggested that mAChRs may act via the $G_{\beta\text{-}\gamma}$ subunits themselves, as well as via G_{α} subunits (reviewed by Caulfield, 1993). thus it appears that activation of phospholipase C (PLC), adenylyl cyclase (AC), as well as the cardiac K^+ channels may be produced by $G_{\beta\text{-}\gamma}$ subunits, but the physiological role of this route, as well as its importance relative to the G_{α} route, has yet to be established.

Coupling to a receptor causes the G protein to release GDP and bind GTP. The activated G protein can regulate the function of an effector, hydrolysis of bound GTP to GDP terminates the regulatory effects of the G protein (Bourne, 1986). The M_1, M_3 and M_5 receptors activate PLC via a pertussis toxin-insensitive G protein. these subunits are thought to mediate primarily excitatory synaptic transmission. M_2 and M_4 receptors inhibit AC activity via a pertussis toxin-sensitve G protein and mediate primarily inhibitory synaptic transmission (McKinney & Coyle, 1991; Wei *et al.*, 1994)

Coupling of G proteins to these enzymes (probably via the third intracellular loop between TM-V and TM-VI, see figure **1.3B.**) results in the generation of second messengers (reviewed by Caulfield, 1993), either cyclic adenosine monophosphate or inositol triphosphate and diacylglycerol, respectively, which in turn may alter the activity of protein kinases. Phosphorylation by protein kinases causes receptor desensitisation and alters the open or closed state of a number of ion channels. G proteins are able to bind directly to K^+ channels without the intervention of any second messenger. In central neurons, activation of mAChR increases the K^+ membrane conductance resulting in hyperpolarisation and so inhibiting ACh release from presynaptic cells due to reduced action. Alternatively mAChR activation may lead to cell depolarisation due to inhibition of cardiac-like K^+ conductances (M-currents).

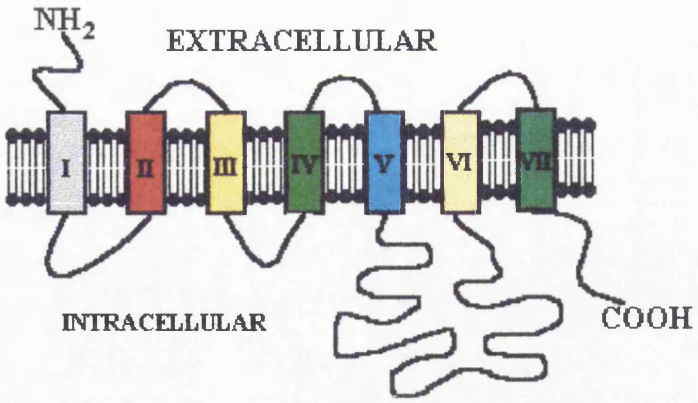


FIGURE 1.1. The muscarinic receptor arrangement in the lipid bilayer showing seven hydrophobic transmembrane spanning regions (TM I-VII) and the extracellular N-terminal and intracellular C-terminal.

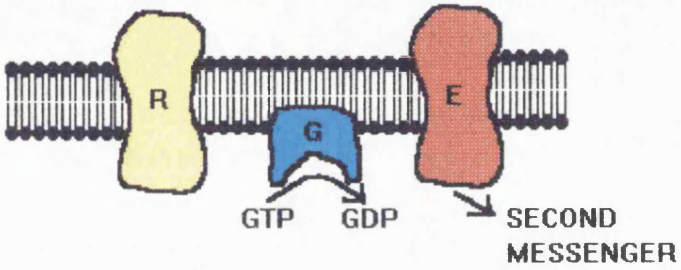


FIGURE 1.2. A schematic representation of the arrangement of a second messenger receptor mechanism in a membrane. R: receptor; G: G protein; E: effector.

1.3.1.2. Localisation of mAChRs

In the CNS the presynaptic localisation of the mAChR has been well documented based on release studies. Muscarinic agonists (oxotremorine, carbachol) reduced electrically- or high K^+- evoked release of ACh from synaptosomes (Nordstrom & Bartfai, 1980; Marchi *et al.*, 1981; Raiteri *et al.*, 1984; Vickroy & Cadman, 1989) and slices (Szerb & Somogyi, 1973; Hadhazy & Szerb, 1977; Weiler, 1989; Marchi & Raiteri, 1989) whereas the muscarinic antagonist, atropine restored or enhanced ACh release. Thus there is a documented, presynaptic inhibitory effect on ACh release.

However binding studies with the muscarinic antagonist [^{3}H]QNB suggested that there were only postsynaptic mAChR, as there was no reduction in binding after lesion in the medial septal nucleus (Yamamura & Snyder, 1974). In 1982, Bowen & Marek showed that the classic muscarinic antagonists (QNB, atropine and scopolamine) had a 10 fold lower affinity for the presynaptic mAChR than for the postsynaptic receptors. This suggests that the low concentration of QNB (1 nM) used by Yamamura & Snyder may have been too low to detect a presynaptic mAChR effect. Using [^{3}H]dexetimide, a muscarinic

antagonist, Consolo *et al* (1984) reported a 20 % reduction in receptor binding after septal lesioning supporting the view of a presynaptic mAChR.

1.3.1.3. mAChR Subtypes

The classical approach to receptor classification has been the discovery and characterisation of selective antagonists and agonists. In the case of the muscarinic receptors, this has also provided the evidence for the existence of subtypes, but their relatively low selectivity, allied to the large number of subtypes, has led to some confusion as regards the definition of the receptor subtypes.

Historically, the first cardioselective muscarinic antagonist to be discovered was gallamine (which is also a nicotinic antagonist). More recently other cardioselective antagonists that lack nicotinic actions: AF-DX 116, himbacine, and methoctramine, have been described. All these antagonists are more potent on cardiac receptor than the receptors on smooth muscle that mediate contraction. The reverse selectivity is shown by 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) and hexahydrosiladifenidol. A different selectivity is shown by pirenzepine, the drug which in the late 1970s and early 1980s, had a major role in the appreciation of the existence of mAChR subtypes. Pirenzepine binds and acts selectively on a muscarinic subpopulation found in neural tissue while it has a lower affinity for the receptors found in heart and smooth muscle (Hammer *et al.,* 1980). Because of the lack of highly selective muscarinic drugs, the results of binding, pharmacological and molecular studies must be combined to arrive at a robust definition of mAChR subtypes. The appeal of such an approach adopted by Hulme *et al.,* in their 1990 review, is that it may be possible to define a muscarinic receptor subtype in terms of its amino acid sequence, pharmacological profile (in binding and functional studies), and its ability to activate selectively one effector mechanism. Table **1.1.** summarises the different mAChR subtypes.

Pharmacological characterisation					
Subtype	M_1	M_2	M_3	-	-
Other names used previously	$M_1\alpha$, A	$M_{2\alpha}$, cardiac M_2, C	M_2, $M_2\beta$, B glandular M_2	M_2	
Selective antagonists	Pirenzepine Telenzepine	AF-DX 116, himbacine, methoctramine gallamine	p-Fluorohexahydro-siladifenidol, Hexahydro-siladifenidol	-	-
	$M_1 > M_3 \geq M_2$	$M_2 > M_1 \geq M_3$	$M_3 > M_1 \geq M_2$		
Molecular characterisation					
Sequences	m1	m2	m3	m4	m5
Other names used previously	mAChRI, M1	mAChRII M2	mAChRIII M4	mAChR IV M3	
Numbers of amino acids	460	466	589/590	478/479	531/532
G protein coupling via:	IP_3	AC	IP_3	AC	IP_3

TABLE 1.1: MUSCARINIC RECEPTOR NOMENCLATURE (as recommended by the 4th Symposium on Subtypes of Muscarinic Receptors and the British Pharmacological Society Nomenclature Subcommittee, 1989). IP_3: triphosphoinositide; AC: adenylyl cyclase.

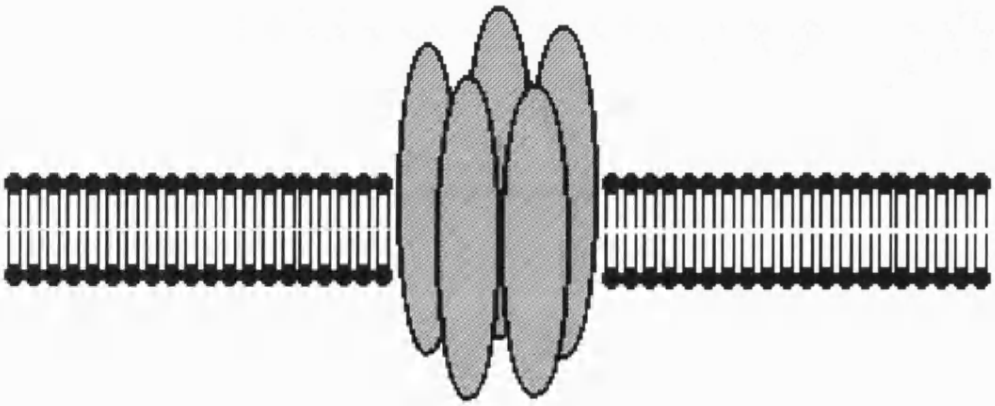
1.3.2. NICOTINIC RECEPTORS

The superfamily of ligand-gated ion channels (LGIC) which mediate rapid excitatory and inhibitory synaptic transmission in the nervous system includes the muscle nAChR, the neuronal nAChR , the γ-amino butyric acid ($GABA_A$) receptor, the glycine receptor, and the $5HT_3$ receptor,. Binding of agonist to these receptors induces the rapid opening of their ion channels, which are selectively permeable to either cations or anions, thereby altering the membrane potential of the cell.

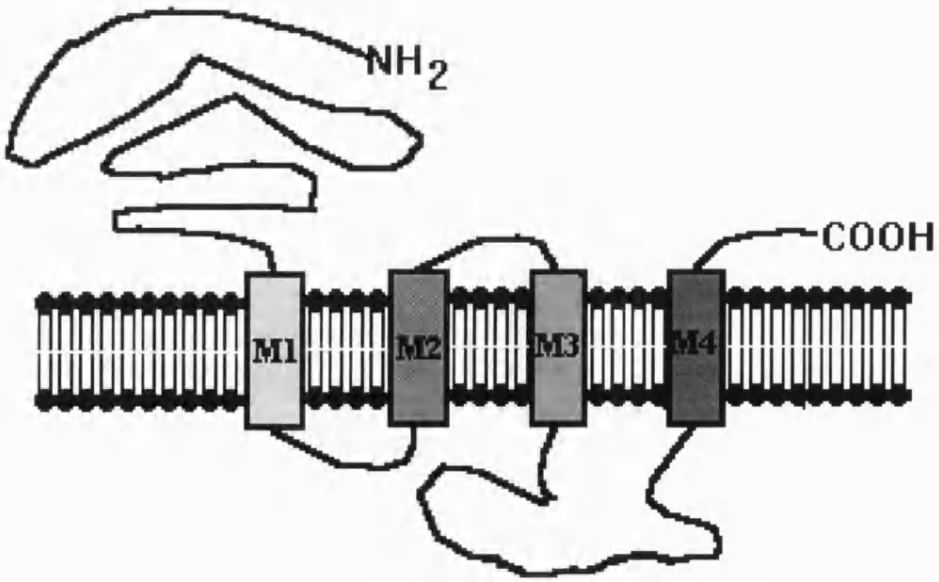
The best characterised nAChR is that obtained from the electric organs of *Torpedo spp.* and *Electrophorus electricus*. The receptor has been extensively characterised, serving as a model for the study of the structure, function and modulation of LGICs (Changeux, 1990). The nAChR is a 250-kDa pentameric complex of four types of subunits in a

stoichiometry of $\alpha_2\beta\gamma\delta$. Each subunit is an integral membrane protein, and the five subunits have been proposed to be arranged around a central pore (figure **1.3A**). The receptor has two ACh binding sites, one on each α subunit, which when occupied by ligand triggers the opening of the channel, allowing permeation by cations (Changeux, 1990). The structure and functional characteristics of the nAChR have been further elucidated by molecular cloning and sequencing. The subunits are highly homologous proteins, each consisting of a large extracellular NH_2-terminal region and four transmembrane domains (M1-M4) as shown in figure **1.3B.** The ACh binding site is located on the extracellular NH_2-terminal domain of the α subunit whereas the channel pore is believed to be formed by the α-helices of the M2 membrane-spanning region of the five subunits (see figure **1.3C.**; Changeux, 1990).

A



B



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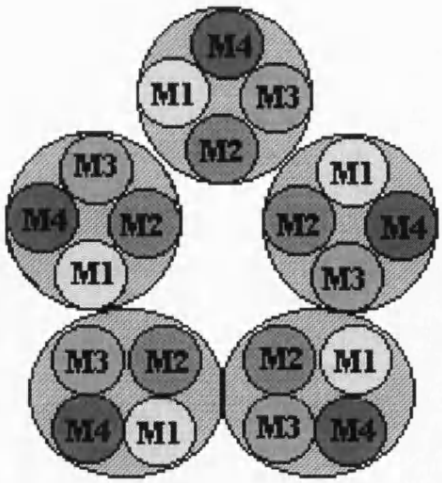


FIGURE 1.3. Schematic model of the topology of an idealised nAChR. A) pentameric subunit structure viewed through the plane of the membrane. B) Membrane topology, viewed through the plane of the membrane of an individual subunit containing transmembrane domains M1-M4. C) Schematic model representing the arrangement of the transmembrane α-helices within each subunit around the central ion channel.

1.3.2.1. Molecular Biology of nAChRs

Biochemical studies demonstrate that the nAChR from rat and chick brain are 300-kDa complexes, initially thought to be composed exclusively of two types of subunits, α and β (Lindstrom *et al.*, 1987; Halvorsen & Berg, 1990), inevitably, the situation has turned out to be more complex with the discovery of α3β4α5 nAChRs in chick ciliary ganglia (Conroy *et al.*, 1992; Vernallis *et al.*, 1993) and homo-oligomeric α7 nAChRs (Couturier *et al.*, 1990; Seguela, *et al.*, 1993). Recombinant DNA technology has resulted in the identification of ten genes in rat and chicken neural tissue that are homologous to muscle nAChR genes (table **1.2.** adapted from Sargent, 1993). These genes encode polypeptides with four hydrophobic, putative transmembrane domain in the approximate relative positions of their muscle counterparts (Figure **1.3.**). The overall amino acid homology between the products of these genes from the same species is 40-55% (table **1.2.**) This homology approaches 100% in the putative transmembrane regions (especially M1-M3) and in selected stretches of the N-terminal extracellular domain, whereas the amino-acid sequence in the putative cytoplasmic segment between M3 and M4 is divergent. All genes code for a protein with two cysteines separated by 13 residues that align with cysteines 128 and 142 of the *Torpedo* α subunit. The deduced gene products fall into two classes, based on whether they have adjacent cysteine residues at positions 192 and 193 (*Torpedo* α numbering) that are found in all α subunits from muscle and electric organ and that are affinity alkylated by the agonist bromoacetylcholine (BAC) and by the antagonist

4-(N-maleido)benzyltrimethlammonium (MBTA). Seven of the genes code for polypeptides which have adjacent cysteines, and these are assigned to the α class; three do not (non-α or β). The muscle α gene is designated α1, and the seven neuronal α subunits are designated α2- α8, more or less in order of discovery. The α subunits themselves fall into two classes, based on subunit homology: α7 and α8, which encode α-Bgt-binding components, have substantial amino acid homology with one another, but less homology with other α subunits than they have with each other (table **1.2.**).

The neuronal non-α subunits are named nα1-nα3 (nα for non-α) in chicken and β2-β4 in rat. For clarity and ease of comparison the terms β2, β3, and β4 will be employed here for both species. Rat and chicken genes of the same name are highly homologous (>70% amino acid identity). The β designation does not mean that these gene products most closely resemble muscle β genes (see table **1.2.**), because they do not. At least in rat, however, the β2 and β4 gene products can substitute for the muscle β1, but not for the α1, γ, or δ gene products, in generating functional, muscle-like nAChRs in *Xenopus* oocytes following injection of mRNAs (Deneris *et al.*, 1988). Based on sequence information alone, there is no reason to classify the three β subunits into a single group, since they are as different from each other as they are from the α subunit. Based on heterologous expression studies, however, at least β2 and β4 belong together because either can encode for a functional nAChR when coexpressed with α2, α3, or α4 (see section **1.3.2.2.**).

SUBUNIT	PROBE	MATURE PEPTIDE M_R # AMINO ACIDS	CONSENSUS N-LINKED GLYCOSYLATION SITES [A]	CYSTEINES [A]	HOMOLOGY (SUBUNIT/%)
Rat [B]					
α2	chicken α2	55,500 484	29, 79, 185	133, 147, 197, 198	α1/48
α3	mouse α1	54,800 474	24, 141	128, 142, 192, 193	α1/52, α2/58
α4-1 [C]	rat α3, mouse α1	67,100 600	24, 141	128, 142, 192, 193	α1/53, α2/68, α3/59
α5	rat β3	48,800 424	112, 140, 186	127, 141, 191, 192	α1-α4/44-55
α6	PCR	53,300 463	24, 141	66, 128, 142, 200, 201	α1, α2, α4, α5/41, 49, 49, 45; α3/59; β2-β4/43-47
α7	PCR (based on chicken α7, α8)	54,200 480	24, 68, 111	116, 128, 142, 200, 201	α2-α5, β2-β4/31-37
β2	rat α3	54,300 475	26, 141	128, 142	β1/45; α1-α5/44-50
β3	rat α3	50,200 434	26, 141	128, 142	β1, β2/40, 44; α1/45; α2-α4/50-56; α5/68
β4	rat β2	53,300 475	15, 72, 117, 145	132, 146	β1/43; β2/64; β3/44; α1/43; α2-α5/46-52
non-α2 [D]	universal oligo	56,100 499	41, 98, 143, 171	6, 26, 101 158, 172	alternatively spliced β4?
Chicken [E]					
α2	chicken α1, γ	58,100 505	31, 81	135, 149, 199, 200	α1/50 rat α2/77
α3	chicken α2	54,800 474	24, 141	128, 142, 192, 193	α1/53; α2/65 rat α3/83
α4	chicken α2	68,400 599	29, 79	133,147, 197, 198	α1/51, α2/80 α3/66 rat α4/73
α5	linkage to α3 [F]	49,000 425	26, 140, 180	127, 141, 191, 192	α1/45; α-α4/50-55 rat α5/84
α6	chicken β4	54,100 463	23, 140	127, 141, 191, 192	β4/48 rat α6/80
α7	chicken α3	54,600 480	24, 68, 111	116, 128, 142, 190, 191	α1-α6, β2-β4/35-42 rat α7/87
α8	chicken α7	55,200 481	24, 111	116, 128, 142, 190, 191	α7/82
β2	chicken γ	54,000 473	26, 143	130, 144	α1-α4/44-55 rat β2/85
β3	chicken α5	50,100 435	28, 143	130, 144	α5/67 rat β3/82
β4	linkage to α3	53,500 467	26, 115, 143	130, 144	β2/70; α1-α5/44-52 rat β4/74

TABLE 1.2: PROPERTIES OF CLONED AND SEQUENCED NEURONAL nAChR GENES

[A] Consensus n-linked glycosylation sites and cysteines are given only for the n-terminal extracellular domain (amino acids 1~200).

[B] The sequence for the rat α6 gene has not yet been published. A rat α8 gene has not yet been identified.

[C] Two cDNAs were isolated for the α4 gene that were virtually identical over the region of overlap and are likely to arise from alternative splicing.

[D] The rat non-α-2 gene differs from the β4 gene in having a 69 base insertion, as well as two smaller insertions, a deletion and an inversion.

[E] The sequences for the chicken α6 and β3 genes have not yet been published.

[F] The chicken α5 and β4 genes were identified by virtue of their linkage to α3: some recombinant α3 phage contained more restriction fragments recognised by α3 probes than could be accounted for by the presence of α3 alone. the α3, α5, and β4 genes are also linked in the rat and human genome.

αBgt-BINDING nAChRs

In vertebrate brain, nicotinic receptors that recognise α-Bgt with high affinity (α-Bgt-AChRs) are distinct from those that do not. For example, α-Bgt-AChRs do not display high affinity binding for [^{3}H]-(-)-nicotine ([^{3}H]nicotine) (Wonnacott, 1986) unlike, for example, the α4β2 subtype. Schoepfer *et al.*, (1990) demonstrated that the majority of α-Bgt-AChRs in chicken brain contain α7 and/or α8 subunits, because mAbs to α7 and α8 fusion proteins immunoprecipitate a large fraction of the high affinity ^{125}I-α-Bgt binding sites (an α7-specific mAb alone immunoprecipitates >90% of α-Bgt-AChRs). These studies demonstrate that the α7 and α8 gene products represent the principal agonist binding subunits of α-Bgt-AChRs. Immunoadsorption of α-Bgt-AChRs with a mAb to α7 does not precipitate detectable amounts of material recognisable with mAbs to α4 or β2; conversely mAb 270 (β2) immunoadsorption does not precipitate detectable α7- or α8-like immunoreactivity (Schoepfer *et al.*, 1990). This suggests that α7 and α8 subunits do not commonly associate with α4 or β2 subunits to form nAChRs in chicken brain. McLane *et al.*, (1990) showed that peptides unique to α5 bind α-Bgt (as do those from α1, α7, and α8), whereas peptides from the corresponding regions of α2-α4 do not. However, whether α5-specific antibodies can immunoprecipitate ^{125}I-α-Bgt binding sites from brain extracts, remains to be determined.

RADIOLIGAND BINDING AND *IN SITU* STUDIES

Radioligand binding and *in situ* hybridisation have been used to visualise nAChRs labelled with high affinity by [^{3}H]nicotine and bungarotoxin-sensitive nAChRs and corresponding receptor mRNA in rat brain (reviewed in Williams *et al.*, 1994). Those nAChR sites labelled with high-affinity by [^{3}H]nicotine are abundant in selective areas of the cerebral cortex (predominantly layers III and IV), thalamus, interpeduncular nucleus and the superior colliculus, but show a low-to-moderate distribution in hippocampus, and hypothalamus (Clarke *et al.*, 1985). nAChRs labelled by [^{125}I]-α-Bgt, are enriched in the hippocampus, hypothalamus and layers I and IV of the cerebral cortex (Clarke *et al.*, 1985; Clarke, 1992). The distribution of nAChR subunit mRNAs in rat correlates reasonably well with the distribution of high-affinity nicotine/ACh and α-Bgt binding sites (summarised in table **1.3.**).

SUBUNIT	ABUNDANCE	DISTRIBUTION
α4, β2, β4	High	Widespread
α3	Medium	Limited: Brain stem motor nuclei, locus coeruleus, thalamus, autonomic ganglia, medial habenula
α7, α6, β3	Low	Restricted: Hippocampus, thalamus, autonomic ganglia
α5, α2	Very low	Focal: Hippocampus, cortex

TABLE 1.3: RELATIVE ABUNDANCE AND DISTRIBUTION OF NEURONAL nAChR SUBUNITS IN THE MAMMALIAN CNS. Adapted from Williams *et al.*, 1994.

The distribution of the α4β2 subunit combination coincides with the distribution of high-affinity nicotine binding sites in rat brain, supporting the immunoprecipitation experiments of Whiting & Lindstrom (1987), who determined that α4 and β2 subunits associated principally but not exclusively with one another.

In summary, molecular and biochemical approaches have identified a family of nAChR subunits that fall into two classes (ACh binding or α subunits, and β subunits), based on the presence of adjacent cysteines and on reactivity with ACh affinity alkylating agents. Immunoprecipitation studies suggest that many neuronal nAChRs are composed of both α and β subunits (e.g. α4β2), other nAChRs might contain two or more kinds of α subunits (Conroy *et al.* 1992), possibly in addition to β subunits.

1.3.2.2. Functional Expression of nAChR genes

One of the aims of this study was to attempt to identify the nAChR subunits making up the presynaptic hippocampal nAChRs. The functional expression of nAChR genes is of interest if the studies aid in the identification of possible subunits which may combine to form functional receptors. Boulter *et al.*, (1987), Wada *et al.*, (1988), and Deneris *et al.*, (1989) found that *Xenopus* oocytes expressed functional nAChRs after injection of mRNAs from the rat α2, α3, or α4 genes in combination with either the β2 or β4 gene products.

Injection of any one mRNA species alone did not lead to the appearance of functional responses, except α4, which produced small depolarisations in response to high concentrations of ACh in about one third of the trials. The same six combinations of chicken genes are functional when expressed following injections of cDNAs into the oocyte nucleus, with transcription under the control of a heat shock or SV40 promoter (Ballivet *et al.*, 1988; Couturier *et al.*, 1990). These results demonstrate that functional nAChRs can be synthesised from specific combinations of α and β subunits and complement the biochemical findings, which suggest that native nAChRs consist of α/β heteromers.

It was thought until very recently that the α7 gene was exceptional in its ability to encode a functional homo-oligomeric nicotinic channel (Couturier *et al.*, 1990): the authors found that voltage-clamped oocytes previously injected with α7 cDNA respond to nicotinic ligands by producing inward currents. These channels are unusual in the sense that β subunit expression is apparently not needed; barring the existence of endogenous β subunits in the oocyte, these nAChRs must consist of α7 homo-oligomers. Currents from α7 nAChRs are blocked by low concentrations of α-Bgt (IC_{50} = 0.7 nM) and less potently by nBgt (Couturier *et al.*, 1990). Coinjection of β2, β3 or β4 cDNA, along with α7 cDNA, did not result in functional responses with properties any different from those expressed in oocytes injected with α7 cDNA alone. Thus, in oocytes at least, α7 subunits apparently cannot coassemble with known neuronal β subunits to form hetero-oligomers that have functional properties different from those of α7 homo-oligomers. Recently, functional α8 homomers expressed in oocytes have been reported (Anand *et al.*, 1994; Gotti *et al.*, 1994; Gerzanich *et al.*, 1994). Immunohistochemical studies (reviewed in Gerzanich *et al.*, 1994) suggest that these receptors have a prominent role in the chick retina.

The rat β3 gene does not form functional nAChRs when coinjected as mRNA with α2, α3 or α4 genes (Deneris *et al.*, 1989). The rat α5 and α6 genes likewise do not participate in the formation of functional nicotinic channels in oocytes when injected in combination with several other α and β genes as mRNAs (Boulter *et al.*, 1990). These subunits may form functional nAChRs when coexpressed with undiscovered (or untested) subunits (Conroy *et al.*, 1992), or they may be members of another family of ligand-gated channels. Another important possibility is that these gene products require post-translational modifications not made by the oocyte in order to function.

Expression of nAChRs is not restricted to oocytes: Whiting *et al.*, (1991) stably expressed nAChRs in mouse fibroblasts by transfecting them with chicken α4 and β2

cDNAs. The fibroblasts acquire functional responses that are pharmacologically similar to those in chicken brain, where both α4 and β2 are widely expressed (Morris *et al.*, 1990). The responses of the M10 cells stably transfected with chicken α4β2 nAChRs is discussed further in chapter 4.

1.3.2.3. Functional Diversity Among Heterologously Expressed nAChRs

AGONIST POTENCY

Neuronal nAChRs are distinct from muscle nAChRs in agonist potency. For example, suberyldicholine is generally more effective than ACh on muscle nAChRs, whereas ACh is generally more effective than suberyldicholine on neuronal nAChRs (Lukas, 1989). Neuronal nAChRs themselves are quite distinct with regard to agonist potency. Mulle *et al.*, (1991) found that the order of potency for neurons from the rat interpeduncular nucleus was cytisine > ACh > nicotine; for nAChRs on neurons from the medial habenula, it was nicotine > cytisine > ACh. These differences could possibly reflect differences in nAChR subunit composition (Luetje & Patrick, 1991). In 1991, Luetje & Patrick measured the rank order potency for the agonists cytisine, nicotine, ACh and 1,1-dimethyl-4-phenylpiperazinium on nAChRs expressed in oocytes from rat β2 or β4 subunits in combination with α2, α3, or α4 subunits. Each pair-wise injection yielded nAChRs with a unique rank order potency. One of the most surprising findings was that the β subunit which had traditionally been assigned a structural role i.e. providing a structural framework for the α subunits, influenced agonist efficacy as much as the nature of the α subunit. Strikingly, the authors reported that sensitivity to one agonist, cytisine, was almost completely determined by the β subunit: nAChRs containing the β4 subunit were more sensitive to cytisine than to any other agonist tested, whereas nAChRs containing the β2 subunit were quite insensitive to cytisine.

Other studies have, however, produced conflicting results. In the rat interpeduncular nucleus (IPN) at least six nAChR genes are expressed: α2, α3, α4, α5, β2 and β4. The channel conductance of IPN neurons measured by Mulle *et al.*, (1991) 35 pS, is very close to that of the largest class of heterologously expressed α2β2 nAChRs (34 pS: Papke *et al.*, 1989); therefore, the α2β2 combination may be the principal class of nAChRs on IPN neuronal cell bodies. Mulle *et al.*, (1991) found that cytisine was as effective as ACh in eliciting responses from the IPN neurons, which according to the oocyte studies of Luetje & Patrick would seem to rule out a major role for β2-containing nAChRs.

Similar attempts to match native nAChRs with heterologously expressed nAChRs of defined composition fail in the medial habenula and in rat PC12 cells. In the medial habenula, for example, nAChR channel conductance (26 pS) is closest to the larger conductance class of heterologously expressed $\alpha3\beta4$ channels (22 pS), but the agonist rank order potency for habenular nAChRs resembles neither $\alpha3\beta4$ nAChRs nor any other class of heterologously expressed nAChRs raises an important question. Do oocytes faithfully translate mRNAs and modify and assemble their translation products? Results from experiments of muscle nAChRs suggest that oocytes can translate and assemble subunits for muscle nAChRs accurately (reviewed in Lingle *et al.*, 1992). If the oocytes are not at fault, then the failure to match native and expressed nAChRs may mean that additional nAChR genes are yet to be identified or that native nAChRs consist of subunit combinations that have not yet been tested in oocytes. In particular, native nAChRs may consist of more than one α and/or β type, for example the $\alpha3\beta2\alpha5$ receptors reported in chick ciliary ganglia (Vernallis *et al.*, 1993). At least two approaches can be envisioned that could reveal the presence of hetero-α and hetero-β-containing nAChRs. One approach is to inject multiple α and/or β subunit mRNAs into oocytes and search for channels that have properties not seen when only one α and one β are injected. Another approach is to perform sequential immunoprecipitations with subunit-specific antibodies (Conroy *et al.*, 1992).

ANTAGONIST POTENCY

Antagonists have also been useful in distinguishing muscle nAChRs from their neuronal counterparts and in distinguishing among different neuronal nAChRs. Paton & Zaimis (1949) demonstrated that decamethonium (C10) was more effective than hexamethonium (C6) in blocking muscle nAChRs, whereas C6 was more effective in autonomic ganglia. This led to the terms "C10 receptor" (muscle) and "C6 receptor" (neuronal). Currently, several antagonists distinguish between neuronal and muscle nAChRs (Lukas, 1990).

Snake toxins haven proven useful for differentiating between neuronal nAChRs (reviewed in Chappinelli, 1991; Loring & Zigmond, 1988). The sensitivity of heterologously expressed nAChRs to nBgt is dependent upon the nature of the subunit content (Luetje *et al.*, 1990). Incubation of oocytes with 100 nM nBgt for 30 minutes completely blocks ACh-induced currents elicited following washing out of the toxin in

oocytes previously injected with rat α3 and β2 mRNAs; under the same conditions, nBgt is completely ineffective at blocking function of α2β2 nAChRs (the sensitivity of α4β2 nAChRs is intermediate between α2β2 and α3β2 nAChRs). Site-directed mutagenesis studies have shown that the amino acid at position 198 of the α subunit (glutamine in α3, proline in α2) is critically important in determining the difference in nBgt sensitivity between α3β2 and α2β2 nAChRs (Sargent, 1993). All three classes of β2-containing nAChRs were blocked by neosurugatoxin, which seems to be highly effective on all neuronal nAChRs so far tested (Luetje *et al.*, 1990). It is an oversimplification to state that nBgt is an efficacious antagonist at all α3 containing nAChRs, again the β subunit influences the pharmacology; ACh-induced currents elicited from α3β2 nAChRs are blocked by pre-incubation with 100nM nBgt, but those elicited from α3β4 nAChRs are less sensitive (Duvoisin *et al.*, 1989).

For all their evident usefulness, it is becoming apparent that heterologous nAChR expression studies must be treated as one paradigm, rather than the sole and definitive answer, for the advancement of our understanding of the plethora of potential nAChR subtypes. It is too early to say whether the observed pharmacological differences between the expressed nAChRs can be used as diagnostic tools for identifying native nAChRs, at least until a population of native channels is identified as arising from a specific combination of subunits in order to compare their properties with those of heterologously expressed channels.

STOICHIOMETRY

Neuronal nAChRs may have an α:β stoichiometry of 2:3, because their activation is typically characterised by Hill coefficients of about 1.5 and because their size (~300 kDa) is consistent with a pentameric structure. Anand *et al.*, (1991) used ^{35}S-methionine to metabolically label nAChRs expressed following injection of chicken α4 and β2 mRNAs into *Xenopus* oocytes. They found 1.46 times more ^{35}S in β subunits than in α subunits after correction was made for their methionine content. This ratio is very close to the expected value of 1.5 for a stoichiometry of $\alpha_2\beta_3$, which suggests that expressed α4β2 nAChRs are pentamers that contain two α and three β subunits. Comparable results were reported by Whiting *et al.*, (1991) for α4β2 nAChRs stably expressed in mouse fibroblasts. Cooper *et al.*, (1991) concluded that α4β2 nAChRs have two α and three β subunits based on the number of channel conductance populations observed when oocytes express a

mixture of α4, β2, and an α4 or β2 whose putative M2 transmembrane domain has been altered by site-directed mutagenesis. A stoichiometry of $\alpha_2\beta_3$ for native nAChRs would seem likely based on these findings and by analogy with muscle nAChRs, which are pentamers containing two α subunits.

1.3.2.4. Presynaptic nAChRs In Brain

nAChRs in autonomic ganglia are responsible for mediating fast excitation, and it is generally assumed that the same holds true for central nAChRs. Nicotinic responses have been elicited from neuronal cell bodies in several areas of brain, including for example, retina, spinal cord, hippocampus, cerebral cortex and septal nucleus in rats, and the retina and lateral spiriform nucleus in chickens (reviewed in Clarke, 1990; Sargent, 1993). However, definitive evidence that central synaptic transmission is mediated by nAChRs only exists at the motor neuron-Renshaw cell synapse in the spinal cord (reviewed in Nicoll *et al.*, 1990). The presence of functional nAChRs on many neuronal cell bodies suggests that nAChRs mediate postsynaptic nicotinic responses widely in the nervous system; though attempts to document this function have been largely unsuccessful (Brown *et al.*, 1983), awaiting further characterisation of central synaptic pathways.

Although nicotinic responses can be elicited from many different areas of the brain, they are not as prominent as might be expected based on mapping studies. This discrepancy may be explained if many of the nAChRs are located on nerve terminals, from which recordings cannot be directly made. In the rat IPN, Brown *et al.*, (1984) and Mulle *et al.*, (1991) characterised one such set of nAChRs found on the terminals of neurons that arise from the medial habenula. Another approach to the characterisation of the nAChRs is the use of synaptosomes, ensuring that the receptors under study are presynaptic in origin. The best characterised presynaptic action of nicotine concerns the enhancement of dopamine release in the striatum (reviewed by Chesselet, 1984; Rapier, 1988, 1990). Nicotine and its agonists are also able to stimulate the release of both [^{3}H]ACh and [^{3}H]GABA from hippocampal synaptosomes (Wonnacott *et al.*, 1989) and it is thought that these nicotinic autoreceptors on cholinergic terminals may subserve a physiological role in the feedback regulation of ACh release. Hexamethonium-sensitive nicotine- and DMPP-evoked release of ACh from cortical synaptosomes has been reported (Rowell & Winkler, 1984), and a similar phenomenon has been demonstrated in synaptosomes of the myenteric plexus (Briggs & Cooper, 1982). The presence of nicotinic receptors corresponding to [^{3}H]nicotine binding sites on cholinergic terminals in cortical and limbic regions helps to

explain the deficits in these ligand binding sites in the brains of Alzheimer patients (section **1.1.2.2.**).

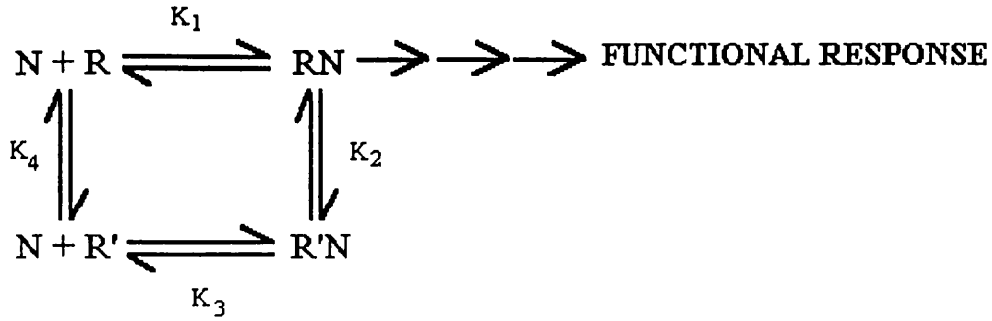
1.3.2.5. Desensitisation of nAChRs

It is well known that high agonist concentrations can cause a desensitisation of nAChRs: functional studies nearly thirty years ago demonstrated desensitisation of ganglionic nAChRs, and the same phenomenon was demonstrated at the beginning of the 1980's in skeletal muscle (reviewed by Ochoa, 1989). More recent studies have reported data which is compatible with the desensitisation of nAChR mediating the release of a variety of transmitters in CNS tissue at much lower agonist concentrations (Rapier et al., 1988; Rowell and Hillebrand, 1992; Grady et al., 1994).

So, in addition to stimulating nAChR channel opening, ACh is able to inactivate channel function by three different mechanisms. Over increasing ACh concentration ranges, these inactivation processes are termed slow desensitisation (seconds to minute time scale), fast desensitisation (milliseconds to seconds), and self inhibition (reviewed in Changeux *et al.*, 1984). Slow and fast desensitisation are thought to be triggered by agonist binding to receptor sites (see below), whereas self inhibition is thought to occur by either a channel blocking mechanism (Sine & Steinbach, 1984) or an allosteric site mechanism (Pasquale *et al.*, 1983).

There is evidence that several processes subsequent to nicotinic receptor activation, such as voltage operated Na^+ and Ca^{2+} channel opening, can also undergo desensitisation (Marley, 1988). In addition, there is evidence that nicotine-induced desensitisation may have several components, depending on whether receptor activation and release occurs (Boksa and Livett, 1984).

The acute desensitisation produced by nicotine and other agonists is accounted for by the two-state receptor model originally proposed by Katz and Thesleff (1957; see also Ochoa et al., 1989). This model is presented below, where **R** represents the ground state receptor, **R'** is the desensitised form, and **N** represents nicotine:



This model predicts two routes leading to nicotinic receptor desensitisation: (a) **R** +**N** ⇔ **RN** ⇔ **R'N** and (b) **R** ⇔ **R'** +**N** ⇔ **R'N**. The first route would involve the **RN** complex and would result in a functional response, whereas the second route does not. The observation that functional responses, such as nicotine-evoked transmitter release from synaptosomes (Grady *et al.*, 1992; 1994; Rowell & Hillebrand., 1994) require low micromolar concentrations of nicotine is consistent with this two-route model where **R** +**N** ⇔ **RN** has an EC_{50} of approximately 1μM. The second route **R** ⇔ **R'** +**N** ⇔ **R'N** has been reported to have an EC_{50} value in the lower nanomolar range, stabilising the desensitised conformation of the receptor without eliciting a response (Bowman *et al.*, 1990; Franke *et al.*, 1992; Wessler *et al.*, 1992; Grady *et al.*, 1994; Rowell, 1994). This process has been termed 'inactivation' rather than desensitisation (Rowell & Hillebrand, 1994) but this is an unnecessary distinction.

Both fast and slow inactivation are believed to involve conformational changes in the receptor (reviewed Ochoa, 1989). Slow desensitisation is known to proceed at ACh concentrations where negligible channel opening occurs and the Hill coefficient for slow rates is 1.0 (Neubig *et al.*, 1982). Consequently, it has been suggested that slow desensitisation occurs from a singly-liganded nAChR state (i.e. only one activating site bound to agonist). Consistent with this scheme, an irreversibly bound agonist analogue, bromoacetylcholine, also blocks agonist binding to desensitised receptors (Damle & Karlin, 1978).

Previous quenched-flow results in both *Electrophorus* (Aoshima *et al.*, 1981) and *Torpedo* vesicles (Hess *et al.*, 1982; Huganir *et al.*, 1986) are consistent with a model where fast desensitisation occurs from a doubly liganded 'pre-open' nAChR state in rapid equilibrium with the open channel state.

The Katz and Thesleff model of receptor desensitisation was modified by Changeux et al., (1984) to take account of the kinetics of desensitisation. In this model four states of the receptor are proposed:

R and **A** = resting and active states, respectively

I and **D** = represent rapidly and slowly desensitised states of the receptor

States **D** and **A** represent ion channel configurations with the highest and lowest ligand affinity, favoured in the presence and absence of ligand respectively. This model differs primarily from the cyclic model proposed in that a rapidly desensitising intermediate state between **R** and **D** is suggested, this state has been substantiated by binding studies using fluorescent agonists (Changeux, 1981), binding studies with the non-competitive channel blocker prehydrohistrionicotoxin (Alberquerque et al., 1974, Dolly et al., 1977), and single channel recordings from embryonic muscle which suggest multiple conductance states (Hamil & Sakmann, 1981).

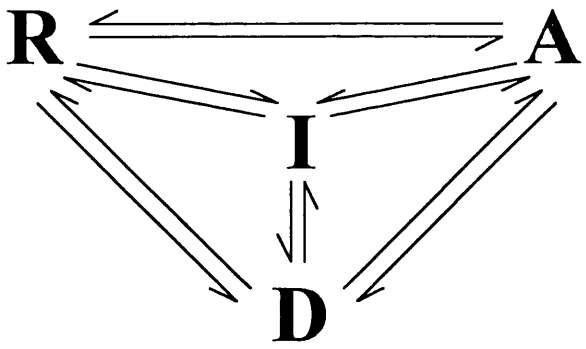


FIGURE 1.4. Schematic diagram of the modified cyclical model to explain desensitisation (Changeux *et al.*, 1984). In this model intermediate states are accounted for, based upon kinetic data. **R** and **A** = resting and active receptor states respectively, **A** has the lowest ligand affinity and is favoured in the absence of ligand. **I** and **D** = rapidly and slowly desensitised states of the receptor, **D** has the highest affinity for the ligand and is favoured in its presence.

The fast component (**I**) acts to increase the rate of ion flux whilst the slow component (**D**) acts to reduce ion flux to undetectable levels (Walker et al., 1982). Patch clamp studies show that nAChR can spontaneously open to the active state (**A**), even in the absence of agonist. This is presumed to be a thermodynamic effect (Jackson, 1984) and is a process accelerated by the presence of agonist (Jackson, 1986).

In quenched flow experiments with *Torpedo* and *Electrophorus* vesicles using high agonist concentrations (50 and 100 mM ACh), Forman & Miller (1988) revealed an nAChR inactivation process occurring before fast desensitisation, which they termed rapid

inactivation. A process which they postulated to be almost certainly the same as the self inhibition by agonists observed in integrated flux experiments (Forman *et al.*, 1987). Forman & Miller (1988) showed that self inhibition/rapid inactivation may occur only after channel activation, suggesting a mechanism in which agonists bind selectively to open channels. This proposed mechanism is consistent with published data that the self-inhibitory potencies of agonists are voltage dependent (Sine & Steinbach, 1984)

1.4. THE CHOLINERGIC HYPOTHESIS

The changes in cholinergic function and transmitter abnormalities detailed in section **1.1.2.**, taken together with evidence for the role of the cerebral cholinergic systems in behaviour taken from other lines of enquiry, have led to the development of the cholinergic hypothesis of dementia in AD (Perry, 1986; Smith & Swash, 1978): the hypothesis which, at the time was called the "single greatest impetus to research into Alzheimer's disease" (Collerton, 1986).

Although there are numerous alternative versions, in its commonest form the cholinergic hypothesis consigns the classical pathological signs of AD to a subsidiary role and states that the loss of cholinergic functions causes or contributes to some of the intellectual impairments particularly the memory deficits of AD (Bartus *et al.*, 1982; Coyle *et al.*, 1983; Perry, 1986; Rossor, 1981).

1.4.1. MODELING ALZHEIMER PATHOLOGY

1.4.1.1. Effects Of Cholinergic Drugs

One strategy used in clinical neuropsychopharmacology is to administer drugs that selectively alter central neurotransmission and then determine whether these manipulations produce symptoms similar to those seen in the disorder. Many studies, for example, have demonstrated that antimuscarinic agents such as atropine and scopolamine can disrupt both the acquisition and performance of a variety of learned behaviours, (Hagan *et al.*, 1988; Aigner & Mishkin, 1986). In contrast, compounds such as physostigmine (PHY) that enhance central cholinergic tone by inhibiting the catabolic enzyme acetylcholinesterase (AChE) can, under certain circumstances, enhance performance in learning and memory tasks, (Aigner & Mishkin, 1986). In addition, at appropriate doses a variety of muscarinic receptor agonists can enhance the performance of animals on tests of learning and memory

(Hagan *et al.*, 1988). This body of research has provided strong evidence that unspecified cholinergic systems in the brain play important roles in forms of learning and memory.

In much of the resultant literature it has become commonplace to discuss the general similarities that exist between the learning and memory impairments seen in AD and those produced in young human subjects by antimuscarinic drugs such as scopolamine, (Bartus *et al.*, 1982). However more recent analyses have raised substantial questions about the adequacy of the scopolamine syndrome as a model for the mnemonic deficits occurring in AD. For example, there is evidence that the specific deficits produced by scopolamine in humans differ substantially from those seen in AD, (Beatty *et al.*, 1986; 1988). In fact, Beatty *et al.*, (1986) concluded that the dissimilarities outweigh the similarities. These results are compatible with other data showing that in young normal subjects the pattern of changes in regional cerebral blood flow produced by scopolamine differs from that seen in subjects with AD, (Honer *et al.*, 1988). To some extent these observations are not surprising because, while muscarinic antagonists produce an acute, short-lasting blockade of cholinergic receptors that are primarily postsynaptic, AD is a chronic, slowly progressing, irreversible disorder that involves, in addition to the documented changes in presynaptic function, substantial pathology of many other neurotransmitter systems, (Gottfries, 1990). Even if cholinergic degeneration was the sole pathological feature of AD, because both muscarinic and nicotinic transmission would be impaired, muscarinic antagonists might not be expected to model the cognitive deficits in this disease. In any event, the behavioural differences that exist between the scopolamine syndrome and AD indicate that if there are specific cognitive impairments due to cholinergic dysfunction in AD, their identity remains unknown. To complicate matters further, Kish *et al.*, (1989) reported that although ChAT activity in the neocortex of patients with dominantly inherited olivopontocerebellar atrophy (OCPA) is reduced to as great an extent as it is in AD, OCPA patients do not show the disabling dementia that is so characteristic of Alzheimer's disease. Importantly though, Kish *et al.*, (1989) noted that in contrast to AD, hippocampal ChAT activity is not reduced in OCPA, raising the possibility that some aspects of cognitive impairment in AD are associated with dysfunction of the septo-hippocampal projection. Equally important, these findings question whether impaired cholinergic function in the cortex contributes to cognitive decline in Alzheimer's disease.

1.4.1.2. Lesions Of Cholinergic Pathways

Studies of the behavioural effects of lesions of cholinergic cells are important because the chronic depression of cholinergic function produced by destruction of cholinergic cells may more closely resemble the cholinergic lesion in AD, secondly because they are a means of examining the role of specific cholinergic pathways in behaviour, and thirdly because they allow cholinergic functions to be examined without the complicating side-effects of drugs. However, such experiments are not without their own difficulties since there is no specific neurotoxin for cholinergic neurons. The early promise of toxins such as AF64A (Walsh *et al.*, 1984) has yet to be fulfilled (Jarrard *et al.*, 1984). Thus, at present, lesions of cholinergic pathways cause variable amounts of collateral damage. It is not yet clear how much of the behavioural deficits which follow lesions of areas containing cholinergic cells are caused by this.

The many cholinergic cells in the mammalian brain may be classified as either intrinsic, local circuit neurons such as those found in the striatum and neocortex, or extrinsic neurons which provide projection pathways innervating distant areas of brain (section **1.2.1.**). Of these latter group of neurons, two have so far attracted major interest for their role in behaviour. These are: (i) the cholinergic cells of the nBM which provide a specific topographical innervation of the neocortex and the amygdala, and (ii) the cells of the medial septal area which, together with the cells of the vertical nucleus of the diagonal band of Broca, provide the cholinergic innervation of the hippocampus (figure **1.1.**).

In AD, both these projection pathways appear to be affected to varying degrees (section **1.1.1**). Combined lesions of these pathways may therefore provide a useful animal model of AD (Overstreet & Russell, 1984). Combined lesions of the medial septal area/nBM lesions disrupt performance in the radial arm maze (Knowlton *et al.*, 1985) though the significance of this is not clear since both media septal and nBM lesions alone cause poor performance in the same task (Hepler *et al.*, 1985; Olton *et al.*, 1984).

Lesions Of The Nucleus Basalis Of Meynert

Electrolytic or neurotoxic lesions of the nBM in the rat bring about the degeneration of the cholinergic neurons constituting approximately 90% of its neuronal population (Woolf *et al.*, 1986), which projects to the cortex. Their destruction is followed by a large decrease in cortical cholinergic parameters such as ChAT activity, and high

affinity choline uptake (HACU). However, the cells of the nBM in the rodent do not form a discrete nucleus as they do in primates but instead are spread throughout the ventral pallidum. Thus, if sufficient cells are destroyed to have a substantial effect on central cholinergic function, large areas of the ventral pallidum are also damaged. The behavioural effects seen after lesions of the nBM may therefore reflect collateral pallidal damage (Flicker *et al.*, 1983; Wishaw *et al.*, 1985) or, depending upon the lesioning technique, disruption of fibres of passage. Fine *et al.*, (1985) have reported that although lesions of the nBM cause both acquisition and retention deficits in passive avoidance learning, only the retention deficits are reversed by grafting cholinergic cells onto the neocortex. Thus it is possible that the acquisition deficit may be due to non-cholinergic or pallidal factors

Lesions Of The Medial Septal Area

Fimbria transection is the standard technique used to disrupt cholinergic innervation to the hippocampus. Lesions result in marked reductions of several presynaptic cholinergic parameters. For example, Araujo *et al.*, (1993) reported reductions in the following areas: ChAT activity (50%), [^{3}H]ACh synthesis (59%), and basal and veratridine (1 μM)-evoked [^{3}H]ACh release (by 44 and 57%, respectively). It is worth bearing in mind that a similar problem to that seen with nBM lesions occurs with attempts to remove the cholinergic innervation of the hippocampus. If all cholinergic afferents are interrupted by lesioning the fimbria-fornix, other hippocampal fibres are also destroyed. Conversely, if smaller medial septal lesions are made, only part of the cholinergic innervation is disrupted. Although the effects of these various lesions are well characterised, the specific role of the cholinergic innervation of the hippocampus remains controversial (Gray, 1982), although it is clearly necessary for performance upon complex tasks (Dunnett *et al.*, 1982; Gage *et al.*, 1984).

1.4.1.3. Comparison Of Lesions And Pharmacological Intervention With Respect To The Effects Of Cholinergic Drugs

There have been few direct comparisons of the effects of cholinergic drugs and lesions of cholinergic cells. Atropine and nBM lesions have similar effects upon performance in the Morris water maze (Wishaw *et al.*, 1985), while medial septal and nBM lesions have effects upon delayed matching to position which, when combined are similar to the effects of scopolamine (Dunnett, 1985).

In summary, the investigation of the behavioural effects of lesions of cholinergic cells is still in its infancy. While lesions of cholinergic nuclei produce performance deficits on behavioural tasks, it is not obvious how these deficits relate to the deficits caused by anticholinergic drugs, or how they relate to the symptoms of Alzheimer's disease.

The Effects Of Cholinergic Dysfunction In Normal Subjects, Compared With The Symptoms Of Alzheimer's Disease

Attempts to draw parallels between the behavioural signs of AD and the effects of manipulating cholinergic function have to surmount several obstacles. These fall into three groups; the difficulties already described in defining the effects of depressing cholinergic function in normals, the problems of defining the symptoms of AD, and the problems of comparing the two.

It is not easy to accurately define the symptoms of AD. Clinically, the disease tends to be diagnosed by exclusion. There is no definite diagnostic pre-mortem test other than cerebral biopsy. Thus any randomly selected group of AD patient is liable to be extremely heterogeneous and any homogeneous group is liable to be unrepresentative of the general run of patients.

Since there is no agreement upon how the effects of anticholinergic drugs or lesions of cholinergic cells should be interpreted, comparisons with AD patients on the basis of concepts such as 'memory' or 'attention' are not currently possible at anything other than the most superficial level. The more limited comparison of whether depressing cholinergic function causes performance deficits similar to AD is all that can presently be achieved.

1.5. TECHNIQUES USED FOR STUDYING TRANSMITTER RELEASE AND ITS MODULATION

1.5.1. *IN VITRO* METHODS: SYNAPTOSOMES AND SUPERFUSION

1.5.1.1. Synaptosomes

Synaptosomes are isolated nerve terminals prepared by controlled homogenisation of brain (McMahon & Nicholls, 1991). The axon is torn off the terminal, which reseals and

remains bioenergetically competent for several hours, showing high respiratory control (Kauppinen & Nicholls, 1986), maintaining a plasma membrane potential of -60 to -80 mV in low K^+ medium (Scott & Nicholls, 1980) and a cytoplasmic free Ca^{2+} concentration of 0.1-0.2 μM (Ashley, 1986; Tibbs *et al.*, 1989; McMahon & Nicholls, 1991a) and releasing neurotransmitter by mechanisms which respond to the same inhibitors and modulators as more complex preparations.

Synaptosomes are capable of releasing neurotransmitter in a Ca^{2+}-dependent manner when depolarised with high K^+ concentrations (Briggs & Cooper, 1982; Rapier *et al.*, 1989) or electrically (DeBelleroche & Bradford, 1973) and contain all the machinery for transducing a depolarising stimulus into an influx of Ca^{2+}, inducing synaptic vesicle exocytosis (McMahon & Nicholls, 1991b). *De novo* transmitter synthesis (Birch & Fillenz, 1985), and the ability to accumulate exogenous transmitter (Bogdanski *et al.*, 1968), are also functions conserved within these structures. The synaptosome can, thus, be used to study the integration of metabolism, mitochondrial and plasma membrane ion circuits and the machinery for the uptake, storage and exocytosis of neurotransmitters.

Although brain slices, like synaptosomes are also capable of respiration for several hours after preparation, their use in studies on presynaptic function is complicated by their histological integrity. Drug diffusion through slices can be a problem, and any presynaptic effect may be indirect, mediated via interconnections with other neuronal or non-neuronal elements. Synaptosomes permit direct access of drug, and though devoid of axons and cell bodies which may normally influence neuronal activity, they provide a simple system for examining nerve terminal function in a precisely controlled manner, an advantage they hold over *in vivo* techniques.

1.5.1.2. Superfusion

Over the last four decades, a variety of techniques has been employed to study neurotransmitter release within the CNS, including superfusion of synaptosomes. A number of apparatuses for superfusion of synaptosomes have been reported in the literature that display various capabilities and limitations (Raiteri *et al.*, 1974; Mulder *et al.*, 1975; Redburn *et al.*, 1975; Collard *et al.*, 1981; Klaff *et al.*, 1982; Drapeau & Blaustein, 1983; Minnema & Michaelson, 1985; Pearce *et al.*, 1991). The continuous superfusion of synaptosomes allows for direct examination of neurotransmitter release from nerve endings and varicosities and has a number of advantages over static incubation systems. This

approach can obviate problems associated with accumulation of neuroactive species and provide superior temporal resolution of transmitter release. Preparations derived from brain tissue, such as synaptosomes are replete with neuroactive species, many of which accumulate in the incubation medium, particularly under depolarising conditions. Accumulation of endogenous neuroactive species in incubations with brain synaptosomes is sufficient to alter transmitter release and presynaptic receptor function (Nordstrom & Bartfai, 1980; Pearce *et al.*, 1986; Pedata *et al.*, 1986). The facilitatory effect on transmitter release produced by addition of receptor antagonists to static incubations of brain synaptosomes also provides evidence of the significant concentrations of endogenous receptor agonists achieved in these incubations (Nordstrom & Bartfai, 1980). In an analogous manner, accumulation in the 'biophase' about nerve terminals in intact tissue preparations, such as brain slices, has been used to demonstrate autoreceptor by numerous laboratories (Suzuki *et al.*, 1988; Weiler, 1989). Clearly, accumulation of endogenous neuroactive species can represent a significant confounding problem to investigators who wish to study the actions and underlying mechanisms of experimental manipulations and agents that modulate neurotransmitter release.

The principle of superfusion is that synaptosomes, brain slices or minces, often preloaded with radiolabelled transmitter or precursor, are continuously superfused with a balanced salt solution, containing glucose to maintain metabolic activity. The effect of drugs introduced into the system, can be monitored by the release of labelled transmitter. The justification of using slices or synaptosomes is that in both systems the extracellular environment of the tissue can be altered, an ability which is hampered in *in vivo* studies of the brain, where the blood-brain barrier severely limits the ability to alter ion and metabolite concentrations surrounding brain cells, and makes quantifying any changes very difficult. Superfusion of synaptosomes, while being less representative of the *in vivo* situation in the CNS than slices, allows the study of presynaptic effects without the complicating range of postsynaptic influences present in slices. Although superfusion systems originally were employed to obviate the confounding effects of transmitter reuptake, the importance of this approach to minimising the effects of endogenous species by prompt removal of the superfusate has been recognised (Raiteri, *et al.*, 1974).

1.5.2. *IN VIVO* METHODS: MICRODIALYSIS

The very qualities of synaptosomal superfusion that make the technique useful for studying neuronal receptors in terms of being a relatively simple system, divorce it from the

in vivo situation and it is often pertinent to make a comparison between the two. There is an increasing need for more accurate and detailed information concerning the complex biochemical reactions in the normal and abnormal brain. The ideal method must provide instantaneous and reliable concentration measurements of a wide variety of interstitial substances in discrete brain areas. Furthermore, normal tissue structure and metabolism should be left unchanged following a particular experimental procedure. None of the available techniques fulfils these requirements. Chapter 6 details the various *in vivo* methods for studying transmitter release and its modulation, with a particular emphasis on *in vivo* microdialysis.

1.6. SUBSTANCE P AND ALZHEIMER'S DISEASE

Although the most consistent peptidergic abnormality which has been described in Alzheimer's disease is a loss of somatostatin in the cerebral cortex of Alzheimer patients, reductions in a number of other common neuropeptides have also been reported (Beal & Mazurek, 1987). Reductions of substance P (SP) in AD brain have been reported at 20-30%, in areas such as the hippocampus, cortex, and temporal lobe and indeed SP has been directly localised to approximately 5% of the neuritic plaques themselves (Armstrong & Terry, 1985).

The primary structure of the eleven amino acid peptide, SP was determined 1971 (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), and this knowledge facilitated studies on its putative role as a neurotransmitter or neuromodulator (Nicoll *et al.*, 1980). Within the CNS, SP has been localised in various nerve fibres (Hokfelt *et al.*, 1980) and in cellular organelles from nerve endings (Schènker *et al.*, 1976). SP is released from nerve tissue within the CNS by depolarising stimuli (Schenker *et al.*, 1976) and can alter the activity of certain neurons when applied in their vicinity (reviewed in Nicoll *et al.*, 1980).

SP belongs to a family of structurally related peptides, the tachykinins. The peptides in this family exhibit biological activities similar to those of SP and share a common COOH-terminal sequence, Phe-X-Gly-Leu-Met-NH_2, the COOH terminal residue of which is amidated, The NH_2-terminal sequence is unique for each peptide and accounts for its specific action.

One of the hallmarks of Alzheimer pathology is the deposition of β-amyloid peptide in the neuritic plaques. Exposure of primary hippocampal neurons to β-amyloid causes

neuronal degeneration, giving rise to the hypothesis that β-amyloid may cause neuronal degeneration when it accumulates abnormally in AD (Kowall *et al.*, 1991). The biological effects of β-amyloid are mediated by an internal peptide sequence (amino acids 25 to 35) that is homologous to the tachykinin family (Yankner *et al.*, 1990), and SP was found to inhibit the effects of β-amyloid on cultured hippocampal neurons in a dose dependent manner, when coinjected intracerebrally, and also administered systemically (IP injection), (Kowall *et al.*, 1991). These observations may form a basis for neuropeptide therapy in Alzheimer's disease, with the ongoing development of non-peptide tachykinin ligands (McLean *et al.*, 1993).

1.6.1. SUBSTANCE P AND nAChRs

In addition to its direct participation in Alzheimer's disease, SP is of interest in this study since it has been shown to colocalise with ACh in the neocortex (Vincent *et al.*, 1983), modulate nAChR-mediated transmitter release, bind to the nAChR directly, and facilitate nAChR desensitisation, (see below).

1.6.1.1. Effects On Transmitter Release

Both inhibitory and facilitatory actions of SP on nicotinic responses have been reported (Livett & Zhou, 1991). First, inhibitory actions: Belcher & Ryall (1977), and Krnjevic and Lekic (1977), showed that in cat spinal cord that electrophoretically administered SP inhibits the ACh-evoked nicotinic activation of Renshaw cells, but has no effect on the excitation caused by either muscarinic agonists or excitatory amino acids. At the frog neuromuscular junction, Steinacker (1977) showed that SP (10^{-6}-10^{-4} M) exhibits an initial phase of synaptic inhibition and then a later phase (60-180 min) of synaptic facilitation. At the Mauthner fiber-giant fibre synapse in the hatchetfish, SP shows predominantly presynaptic and postsynaptic inhibitory action (Steinacker *et al.*, 1976) and, in the rat and chick sympathetic ganglia, SP inhibits the nicotinic depolarisation of ganglionic neurons (Akasu *et al.*, 1983) In isolated bovine adrenal chromaffin cells, SP inhibits the ACh- and nicotine-evoked release of catecholamines, but not that evoked by K^+, veratridine or muscarinic agonists (Livett *et al.*, 1979; Role *et al.*, 1981; Higgins & Berg, 1988).

In addition, SP enhances ACh-induced desensitisation and reduces the number of bursts evoked by ACh (Clapham & Neher, 1984). In PC12 cells, SP inhibits the increase in

nicotinic agonist-induced $^{23}Na^{+}$ influx and enhances the desensitisation of this nicotinic response (Simasko *et al.*, 1986; 1987).

Facilitatory actions of SP on the nicotinic response: in addition to the aforementioned inhibitory actions, SP shows facilitatory actions in a number of neural and endocrine systems. In isolated bovine chromaffin cells, SP (10^{-5} M), present together with high concentrations of ACh or nicotine, completely protects against nicotinic desensitisation (Livett & Boksa, 1984; Boksa & Livett, 1984). SP also protects against desensitisation of $^{45}Ca^{2+}$ uptake into these cells (Boksa, 1985). The ability of SP (10^{-6} M) to exhibit inhibitory or facilitatory effects on catecholamine secretion depends on the concentration of nicotinic agonist used (Khalil *et al.*, 1988).

1.6.2.2. Effects On Nicotinic Agonist Binding To nAChRs

Min & Weiland (1993), studied the effects of SP on the binding of agonists to the nAChR of torpedo electroplaque. They reported that the peptide had no effect on ^{125}I-α-Bgt and did not bind to the high affinity local anaesthetic site, but increased the apparent affinity of the cholinergic agonists carbamylcholine and acetylcholine at equilibrium. The effect of SP on the equilibrium binding of [^{3}H]ACh was examined directly, and the peptide appeared to increase the affinity of the binding of the second molecule of agonist, with no effect on the binding of the first molecule of ACh. This result indicates that SP can affect the co-operative interactions between agonist binding sites. In the same study, SP (Min & Weiland, 1993) also appeared to increase the rate of carbamylcholine-induced desensitisation. In a parallel study, Min *et al.*, (1994) examined the binding of tritiated SP to nAChR-enriched *Torpedo* electroplaque membranes. The authors found that SP interacted directly with two populations of binding sites on the nAChR. The lower affinity sites (K_d = 25 μM) would appear to reflect interactions either with the receptor, perhaps at the protein-lipid interface, as proposed for the low affinity sites of local-anaesthetic-type blockers (Heidmann *et al.*, 1983), or with components of the membrane. The high affinity component of binding (K_d = 0.55 μM), which is seen only in the presence of agonist and of which there is only one site for each receptor, is likely to represents the functionally relevant interaction with the receptor. The binding affinity of the agonist-induced high affinity binding site was significantly different from the SP binding to G protein-coupled receptors (Min *et al.*, 1994). Interestingly, SP specifically labelled the γ and δ subunits, which, although consistent with a binding site within the ion channel as proposed by Min *et*

al., (1993) means that the relevance of this SP binding site to neuronal nAChRs which lack the γ or δ subunits is questionable.

In summary, there is a large body of evidence suggesting that SP is colocalised with ACh, is involved in the pathology of Alzheimer's disease, and is able to modulate nAChR-mediated transmitter release. The possible role of substance P in the modulation of ACh release by presynaptic hippocampal nAChRs merits examination.

1.7. SUMMARY TO CHAPTER 1

The characteristic pathology of Alzheimer's disease emphasises the importance of the hippocampal cholinergic system in higher cognitive function. Anatomically, this system is slowly being understood and the various sub-cortical brain regions which innervate both the hippocampus and the neocortex are increasingly well documented. On a pharmacological level however, the role of the hippocampal muscarinic and nicotinic acetylcholine receptors, is far from clear and complicated by the still burgeoning heterogeneity of these receptor subtypes. Both nAChRs and mAChRs exist presynaptically, and have been shown to modulate hippocampal ACh release both *in vitro* and *in vivo*. The presynaptic nAChRs which facilitate ACh release, represent an opportunity for therapy of early stage dementia, while the cholinergic anatomy is sufficiently intact to respond to modulation.

The identity of the presynaptic nAChR subtype(s) is unknown, and the paucity of subtype-specific nicotinic ligands precludes any direct identification of the subtype(s) present in the rat hippocampus, but a more complete pharmacological profile is essential before any rational cholinergic drug therapy can be contemplated. An *in vitro* pharmacological profile of the nAChRs should address issues such as agonist and antagonist specificity, propensity of the receptor to desensitise, and modulation of this desensitisation phenomenon by substance P. *In vivo*, the effect of nicotine on ACh release in the rat hippocampus is poorly understood, and a microdialysis study addressing this problem would complement the *in vitro* studies.

1.8. AIMS OF THE PROJECT

The importance of cortical and hippocampal presynaptic nAChRs as therapeutic targets in dementing diseases has been outlined. In the past few years, the advances in

recombinant DNA technology have led to the identification of an increasing number of nAChR subunits which in turn provides for a multitude of potential combinations, suggesting that many functional subtypes of neuronal nAChR are possible (section **1.3.2.1.**). Though information derived from heterologous expression systems combined with *in situ* hybridisation studies has provided *in vitro* model systems in which the pharmacology of compounds acting at putative subtypes of neuronal nAChRs can be evaluated using electrophysiological and biochemical techniques (section **1.3.2.3.**), at the present time the subtype identity of native nAChRs is largely unknown. Much in the way of basic research is needed before the development of a program of rational drug design can be contemplated. As such, the main aim of this project was to probe the pharmacology of the presynaptic hippocampal nAChRs: it was hoped that building up a pharmacological profile with a number of the available nicotinic agonists and antagonists, would yield some clues as to the physiological/pharmacological roles of these nAChRs.

On a practical level, the goals of the present work were:

(a) To build upon the superfusion work of Thorne (1990), and establish a superfusion protocol using the automated Brandell SP-06 and SP-12 machines, suitable for the study of [^{3}H]ACh release from hippocampal synaptosomes.

(b) To build a pharmacologically profile of the presynaptic nAChR in the rat hippocampus using a variety of nicotinic ligands.

(c) To study the effects of repetitive agonist stimulation, and exposure to substance P, on nAChR desensitisation/inactivation.

(d) To investigate the *in vitro* binding of [^{3}H]nicotine and [^{3}H]substance P to rat hippocampal membranes.

(e) To analyse the binding of [^{3}H]nicotine and ChAT activity of rat hippocampal preparations from normal and cholinergically lesioned rats.

(f) To assess the ability of nicotine to evoke ACh release in the hippocampus of freely moving rats using the technique of *in vivo* microdialysis.

MATERIALS

CHEMICALS

[Methyl-^{3}H]choline chloride ([^{3}H]Ch; 80 Ci/mmole; 1 mCi/ml in 95% ethanol) and [Methyl -^{3}H]acetyl Coenzyme A ([^{3}H]AcCoA; 3 Ci/mmole) were obtained from Amersham International (Amersham, Bucks., UK). The [^{3}H]choline was stored at -20°C. The [^{3}H]AcCoA was diluted with Triton X-100 buffer to give a final concentration of 2 mM (specific activity 10 μCi/μmole) and stored at -20°C in 100 μl aliquots.

(-)N-methyl-[^{3}H]nicotine (85.1 Ci/mmole; 1 mCi/ml), and [^{3}H][Sar^{9}, Met$(O_2)^{11}$]substance P ([^{3}H]SP; 32.5 Ci/mmole; 1 mCi/ml) were obtained from NEN. [^{3}H]nicotine was stored at -20°C in a 10-fold concentration of mercaptoethanoic acid to minimise the oxidative degradation of nicotine. [^{3}H]SP was stored at -20°C.

(-)Nicotine hydrogen (+)tartrate was purchased from BDH chemicals (Poole, Dorset, UK). Physostigmine was obtained from Sigma Chemical Company Ltd. (Poole, Dorset, UK). DHβE was a gift from Merck, Sharp & Dohme Research Laboratories (Harlow, Essex, UK). ABT-418 was a gift from Abbott Laboratories, (Abbott Park, Illinois, USA). MLA was a gift from Prof. M. Benn, Dept. Of Chemistry, University of Calgary, (Canada). (+)Anatoxin-a was donated by Dr. E.X. Albuquerque, Department of Pharmacology and Experimental Therapeutics, University of Maryland (Baltimore, USA).

Percoll was obtained from Pharmacia (Milton Keynes, UK).

Optiphase 'Safe' scintillant was used for determination of radioactivity in a Packard 1600 TR liquid scintillation counter, giving a counting efficiency of 50-55%.

All other reagents were obtained from Sigma Chemical Company Ltd (Poole, Dorset, UK), Aldrich (Gillingham, Dorset, UK), or BDH (Poole, Dorset, UK) and were of an analytical (AR) grade.

BUFFERS

ARTIFICIAL CEREBROSPINAL FLUID (aCSF)

Composition in mM: 125 NaCl; 2.5 KCl; 1.18 $MgCl_2$; 1.26 $CaCl_2$. Containing neostigmine (2 μM, Sigma), and stored at 4°C.

KREB'S BICARBONATE

Kreb's bicarbonate buffer used throughout the experimental procedures was freshly prepared and had a final composition (mM): 118 NaCl, 2.35 KCl, 1.2 $MgSO_4.7H_2O$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 2.4 $CaCl_2.2H_2O$ and 10 D-glucose, equilibrated to pH 7.4 using 95% O_2/5% CO_2 for 2 hours at room temperature.

LOWRY SOLUTIONS

2% (w/v) Na_2CO_3 in NaOH.

1% (w/v) $CuSO_4$.

Folin's reagent diluted 1:1 with DDW

PBS

K_2HPO_4 8 mM; KH_2PO_4 2 mM; NaCl 7.5 mM. Fresh solution made up before each assay in double distilled water (DDW), from stocks stored at 4°C, pH 7.5 with NaOH.

TRIS

Tris 50 mM. Stored in the dark at 4°C and pH to 7.4 with dilute HCl before use.

TRIS-Mg^{2+}

Tris 50 mM; $MgCl_2$ 2 mM. Stored in the dark at 4°C and pH to 7.4 with dilute HCl before use.

TRITON X-100

50 mM phosphate (K_2HPO_4; Na_2HPO_4), NaCl 200 mM; EDTA 1 mM; 0.5% Triton X-100. Stored in the dark at 4°C.

METHODS

2.1. THE SUPERFUSION SYSTEM

2.1.1. PERCOLL DISCONTINUOUS DENSITY GRADIENTS.

Percoll filtered through a Millipore AP-15 pre-filter to remove aggregates, was used to form solutions of 23% (v/v), 15%, 10% and 3% Percoll in 0.32 M sucrose. After adjustment of the pH to 7.4 with dilute NaOH/HCl, four step gradients were formed by the sequential layering of 2 ml of each Percoll solution into Sorvall 15 ml polycarbonate tubes. A peristaltic pump with a fine bore needle (25G), set to a slow running speed of 1 ml/min in order to minimise disturbance at the density interfaces, was used for layering. The gradients were stored overnight at 4°C before use, (Thorne *et al.*, 1991).

2.1.2. BRAIN TISSUES

Tissues were derived from adult male Sprague-Dawley rats (250-350 g, Bath University Animal House breeding colony). The rats were killed by cervical dislocation, decapitated, the brains removed rapidly and placed ventral surface down on sucrose-soaked filter paper over ice. The brain stem and cerebellum were removed leaving the forebrain. An incision was made along the length of the longitudinal fissure, separating the cerebral hemispheres, but leaving the diencephalon intact. The neocortex (hippocampus still attached) was peeled back from the diencephalon, and the latter was removed en masse. Typical tissue weights were: 90-100 mg / hippocampus.

2.1.3. SYNAPTOSOME PREPARATION

The subcellular fractionation of hippocampal S1 supernatant on discontinuous Percoll gradients was a described by Dunkley *et al.* (1987; 1988).

Excised tissue was homogenised (10% w/v in 0.32 M sucrose, pH 7.4) in a pre-cooled glass-Teflon homogeniser of 0.31 mm clearance, by 2 x 6 strokes at 200 rpm. The supernatant (S1) formed from a 10 min 1000 g spin, was diluted with sucrose and 2 ml aliquots were layered onto the gradients using a large bore needle (19G) This and all

subsequent centrifugation steps were carried out in the Sorvall RC 5B Superspeed centrifuge (SM 24 rotor). One gradient was used per hippocampus.

After layering, the Percoll gradients were centrifuged for exactly 5 min (excluding acceleration and deceleration times) at 32,000 g. The synaptosome fraction - F4 - at the interface of the 23% and 15% Percoll/sucrose layers was removed with a Pasteur pipette and washed in two resuspension/centrifugation steps prior to the final resuspension. In each step the F4 fractions from 2 gradients were pooled, resuspended in 10 ml of buffer and centrifuged for 15 min at 15,000 g. The buffer used throughout was Kreb's bicarbonate. After the final wash the pellet was gently resuspended in buffer to a protein concentration of approximately 1 mg/ml (for hippocampi from 4 rats, this was approximately 2100 µl).

All centrifugation steps were carried out at 4°C and tissue preparations were held on ice until use, to preserve synaptosomal viability.

2.1.4. PROTEIN DETERMINATION

Protein concentrations were determined using the method of Lowry *et al.*, (1951). Standard curves were constructed using a frozen stock of 1 mg/ml bovine serum albumin (BSA) over the concentration range 0-350 µg/ml.

Duplicate samples (0.2 ml) of BSA standards or F4 synaptosomes, diluted 1 in 5, 1 in 10 with distilled water were incubated with 1 ml alkaline cupric tartrate (freshly prepared by mixing 1 volume 1% (w/v) $CuSO_4.5H_2O$, 1 volume 2% (w/v) sodium tartrate and 100 volumes 2% (w/v) Na_2CO_3 in 0.1M NaOH), at room temperature for 10 min. After incubation, Folin-Ciocalteau's reagent (0.1 ml; diluted 1:1 with distilled water) was added to each sample and the colour allowed to develop at room temperature for 40 min. Absorbance was read at 690 nm in a Titertek Multiscan Spectrophotometer, using distilled water as a blank.

2.1.5. CHOLINE UPTAKE

The F4 synaptosome preparation (1900 µl) was incubated at 37°C for 10 min before the addition of [^{3}H]Ch (100µl: 16 µM, 0.5 mCi/ml: prepared by 1:1 dilution of stock [^{3}H]Ch with 20 µM choline chloride; final assay concentration 0.8 µM). After 30 min at 37°C the mixture was centrifuged in a bench microfuge (1 min, 3000 g). The supernatant

was discarded, removing excess [^{3}H]Ch that had not been taken up by the synaptosomes. The pellet was gently resuspended in Kreb's buffer to 2 ml; triplicate samples (10 µl) were filtered on Whatmann GFC filters pre-moistened with Kreb's buffer in a Millipore manifold filtration unit to determine [^{3}H]Ch uptake. Radioactivity was measured in 3 ml of scintillant.

Samples (12 x 150 µl) of the [^{3}H]Ch-loaded F4 fraction were taken for superfusion.

2.1.6. THE SUPERFUSION PROTOCOL

2.1.6.1. Agonist-Evoked Release

The effects of nicotinic agonists on ACh release were studied using the Brandell SF-06 superfusion system.

The [^{3}H]Ch-loaded synaptosomes (150 µl) were loaded into each of the perfusion chambers. In each case the synaptosome sample was placed on a Whatmann GFF filter, pre-moistened with Kreb's buffer, in the superfusion chamber. Another Whatmann GFF filter was positioned on top of the synaptosomes before connecting the apparatus. Kreb's buffer (maintained at 37°C in a water bath) was pumped through the chamber at a flow rate of 250 µl/min. The superfusate (3 min fractions, 750 µl) was collected into minivials and 3 ml of scintillant added to each before the radioactivity was measured.

After a 45 min washout period, the synaptosomes were stimulated with a 20 s pulse of agonist (made up in Kreb's buffer); two such stimulations were routinely given before a third stimulation with 20 mM KCl (KCl isotonically replacing NaCl) was administered. The agonist pulse was separated from the bulk flow of the Kreb's buffer by 10 s air bubble (Rapier *et al.* 1988). The responses to successive stimulations were designated S1, S2 and S3 and evoked release was quantified by summing the radioactivity in fractions contributing to a peak of release, after subtraction of the baseline (see 3.5. DATA ANALYSIS). This was converted to pmol [^{3}H]ACh released per mg protein, by reference to the specific activity of the [^{3}H]Ch used to load the synaptosomes and the amount of tissue loaded into the perfusion chambers. Results were normalised for variations in [^{3}H]Ch uptake between experiments.

2.1.6.2. Antagonists

The effect of antagonists was examined by introducing the drug into the perfusion buffer three fractions before the agonist pulse and maintaining it throughout the remainder of the experiment. All subsequent agonist or KCl pulses were delivered in Kreb's buffer containing the antagonist.

2.2. RADIOLIGAND BINDING ASSAYS

In all binding assays, the S1 fraction (see section **2.1.3.**) was used as the source of rat brain membranes. This was either prepared on the day of the assay, or stored frozen as aliquots at -20°C, at a protein concentration of 1 mg/ml.

2.2.1. [^{3}H]NICOTINE BINDING

2.2.1.1. General Binding

The equilibrium binding procedure was based on the rapid filtration assay of Romano & Goldstein (1980). Assays were conducted in triplicate in conical bottomed polystyrene tubes to facilitate the small incubation volume of 270 µl. For routine binding, the reaction mixture consisted of 250 µl membrane suspension and 10µl [^{3}H](-)-nicotine (final concentration 20 nM). Non-specific binding was defined by incorporation of 10 µl of a solution of (-)-nicotine bitartrate (final concentration 10 µM). Specific binding was calculated as total binding minus non-specific binding. The reaction was initiated by adding the suspension of membranes with vortex mixing and each sample was incubated at room temperature for 30 minutes, then at 4°C for 1 hour. The reaction was terminated by adding 4 ml of ice-cold buffer and quickly filtering at 4°C under vacuum through Whatmann GF/C glass microfibre filters, followed by 2 x 4 ml washes of buffer. In order to retard the non-specific binding of nicotine, the filters were preincubated for at least 1 hour in 0.3% (w/v) polyethylenimine at 4°C. After filtration, the filters were placed in scintillation vials containing 5 ml Packard Optiphase Safe scintillation fluid, allowed to come to room temperature and then counted in a Packard 1600 liquid scintillation counter. Specific binding was calculated as the difference between total and non-specific binding.

2.3.1.2. Competition Binding

For competition binding assays was used at a final assay concentration of 10 nM. Total incubation volume was 270 µl: 5 µl of [^{3}H](-)-nicotine was added to 5 µl of competing compound (10^{-10}-10^{-3}M) and the reaction initiated by the addition of 250 µl of membrane suspension. For each competing compound, 3-6 experiments were performed on different membrane preparations. For competition assays involving substance P, a cocktail of protease inhibitors (40 µg/ml bacitracin, 2 µg/ml chymostatin and 4 µg/ml leupeptin) were included in the assay buffer to prevent proteolysis of the peptide.

2.2.3. [^{3}H]SP BINDING

2.3.3.1. General Binding

The protocol for neurokinin receptor binding assays was based on the procedure of Quirion & Dam (1993). Briefly the protocol is as follows: 200µl of the hippocampal S1 homogenate was incubated for 90 min at 25°C in 400 µl of a buffer containing 50 mM Tris-HCl (pH 7.4 at 4°C), 3 mM $MnCl_2$, 0.02% BSA to minimise losses of ligand to nonreceptor surfaces, 40 µg/ml bacitracin (Sigma, St. Louis, MO), 2 µg/ml chymostatin (Sigma) and 4 µg/ml leupeptin (Sigma) to ensure the stability of the radiolabelled ligand during the incubation period, and various concentrations of [^{3}H][Sar^9,Met(O_2)^{11}]substance P ([^{3}H]SP) for saturation (0.01 to 50 nM). Nonspecific binding was determined in the presence of 5 µM unlabelled SP. Under such assay conditions, the radiolabelled probe has been reported to be highly stable during incubation (Quirion & Dam, 1993).

Incubations were terminated by rapid filtration through Whatmann GF/C filters presoaked in 0.1 % polyethylenimine (PEI) for at least 3 hours prior to filtration. The use of PEI-treated GF/C filters minimises binding of ligand to filters. Filters were washed 3 times with 3-4 ml of cold 50 mM Tris-HCl buffer (pH 7.4). After filtration, the filters were placed in scintillation vials containing 5 ml Packard Optiphase Safe scintillation fluid, allowed to come to room temperature and then counted in a Packard 1600 liquid scintillation counter. According to Quirion & Dam, (1993), under such assay conditions, [^{3}H]SP apparently binds to a single class of high affinity (K_d 1.4 ± 0.5 nM), low capacity (B_{max} 160 ± 3.0 fmol/mg protein) sites in rat brain homogenates. The same authors report that at concentrations approximating K_d values, specific binding reached rapid equilibrium (< 30 min) at 25°C and represented between 70 and 75% of total binding. The ligand,

[^{3}H]SP, specifically and selectively labels the NK-1/SP receptor subtype since only NK-1 competitors such as substance P and unlabelled [Sar^9,Met(O$_2$)^{11}]SP behave as potent competitors (K_i in low nanomolar range) in this assay whereas selective NK-2 ([Nle^{10}]NKA$_{4-10}$) and NK-3 (senktide) analogues are virtually inactive (Dam *et al.*, 1990).

2.3.3.2. Competition Binding

Competition assays were essentially as described in section **2.3.2.1.**, with the radioligand[^{3}H]SP used at a final assay concentration of 2 nM, and incubated with nicotine concentrations of 0.1 nm -1 μM.

2.3. CHOLINE ACETYLTRANSFERASE

The protocol used to determine ChAT activity was based on the liquid cation-exchange method described by Fonnum (1975). The washed S1 fractions were resuspended in Triton X-100 buffer to give a protein concentration of 1 mg/ml. Samples (10 μl) were incubated for 10 min at 37°C with 20 μl of assay buffer in the presence and absence of formic acid. The assay buffer, freshly prepared, comprised 12.5 mM choline chloride, 0.1 mM neostigmine bromide, 0.2 mM [^{3}H]acetyl-coenzyme A (specific activity 10 mCi/mmol), and 5 mg of bovine serum albumin/ml in phosphate buffer. After 10 min, the reaction was stopped by adding 20 μl of formic acid. [^{3}H]ACh was extracted in 0.3 ml of heptan-2-one containing tetraphenylboron (15 mg/ml). A sample (150 μl) of the upper, organic phase was counted for radioactivity in 5 ml of Optiphase-Safe (LKB) in a Packard scintillation counter. The nonspecific acetyltransferase activity was determined in the presence of bromoacetylcholine (0.1 mM), a ChAT inhibitor.

2.4. MICRODIALYSIS

2.4.1. SURGERY

All animal surgery was carried out by Dr Pete Hutson or staff at the animal surgery unit at Merck Sharp & Dohme Research Laboratories, Harlow, Essex. Animals were maintained in a vivarium on a 12 h light/12 h dark cycle (lights on at 0700) and were provided with free access to food and water. Following surgery, animals were transferred to post-operative care for 2 hours and closely observed. Following recovery, animals were

transferred to clear perspex microdialysis chambers which were sufficiently large to allow the rats to move around freely.

Male Sprague-Dawley rats (250-300 g) were anaesthetised with pentobarbitone (60 mg/kg, i.p., Sagatal, RMB) and implanted with a dialysis probe in the hippocampus (A -4.8 mm from bregma; L 4.8 mm; V 8.0 mm below dura) coordinates according to Paxinos and Watson. The probe was essentially as described by Hutson *et al.*, (1985) except that a single piece of quartz tubing (VS 150/40, SGE) was used instead of two glass capillaries and a membrane length of 5 mm was used.

2.4.2. MICRODIALYSIS ASSAY

Nicotine solutions were prepared by dissolving the tartrate salt in saline (pH always above 5.0), which was administered as subcutaneous injection in the flank; mecamylamine solutions were prepared from the hydrochloride and delivered intraperitoneally (i.p.). Microdialysis was performed in conscious, freely moving animals over a period of 6h. Approximately 18 h following recovery from surgery the probe was perfused with aCSF at a rate of 2 µl/min. Two hours after the start of the experiment, (establishment of the baseline) drug samples were administered and microdialysate samples were collected at 20 min intervals and frozen at -70°C until required for analysis of acetylcholine by HPLC with a post-column enzyme reactor and electrochemical detection essentially as described by Damsma *et al.*, (1988).

2.4.3. HPLC

The system used was a Chromspher 5 µM C18 reverse phase column (100 x 3 mm) as a pre-column to saturate the mobile phase with silica, a Chromspher guard column (10 x 2.1 mm) and a Chromspher 5 µM C18 reverse phase analytical column (100 x 3 mm) pre-loaded with lauryl sulphate (5 mg/ml in mobile phase). The enzyme reactor (10 x 2.1 mm) was loaded with 500µl mobile phase containing 80 units acetylcholine esterase (EC 3.1.1.7 type VI-5 from electric eel, Sigma) and 40 units choline oxidase (EC 1.1.3.17 from Alcaligenes, Sigma). The mobile phase consisted of 0.2M KH_2PO_4 and 0.3 mM EDTA (pH 8.0 with 5M KOH). This was filtered (0.45µm) and degassed with helium before use at as flow rate of 0.6 ml/min without recycling. A BAS LC $B electrochemical detector (platinum electrode set at +0.5 V) was used to detect the evolved hydrogen peroxide.

CHAPTER 3. CHARACTERISATION OF A SUPERFUSION SYSTEM TO STUDY ACETYLCHOLINE RELEASE

3.1. INTRODUCTION

Thorne (1990) had adapted the earlier work of Rapier *et al.*, (1988), and optimised a home-made superfusion system to study nicotine-evoked [^{3}H]ACh release from hippocampal synaptosomes. However, the responses to nicotine, though quantifiable, were relatively low, and it was hoped that the use of an automated apparatus would eliminate much of the variability between superfusion chambers and maximise the sensitivity of the procedure. A Brandell Superfusion SP-06 apparatus was adopted for this purpose.

As shown in figure **3.1A.** the SP-06 is a relatively simple design. An electrical heating block maintains a water bath at 37°C. In the water bath are located the reservoir buffer containing oxygenated Kreb's buffer and also any agonist/KCl solutions. A perfusion pump ensures an constant flow of buffer between the reservoir and the tissue block (shown in detail in figure **3.1B.**). The superfusion buffer is driven upwards through the tissue chamber and the superfusate collected by the fraction collector below. Pulses of drug solution are given by moving the aspirator block from the reservoir buffer into the drug solutions.

In order to characterise superfusion achieved using the Brandell Superfusion SP-06 apparatus, initial studies utilised cortical rather than hippocampal synaptosomes. The cortex was chosen since it is a brain region with good cholinergic innervation (from the nBM, figure **1.1.**) and a comparatively large amount of tissue is obtained from each rat, so consequently fewer rats were sacrificed during the characterisation process.

Many factors needed to be characterised before the Brandell SP-06 could be used routinely to assay responses of the presynaptic nAChR to agonists. In order to achieve maximum sensitivity of the automated apparatus, one of the primary aims of moving up from the home-made rig of Rapier *et al.*, (1988; 1990) or Thorne *et al.*, (1990; 1991), it was necessary to re-examine Thorne's standard superfusion protocol in terms of: amount of protein loaded per chamber, the flow rate of Kreb's buffer, the calcium dependence of the responses to agonist and potassium, and the concentration of potassium used as a standard in each superfusion experiment. Each of these parameters was examined initially

FIGURE 3.1A. SCHEMATIC REPRESENTATION OF THE BRANDELL SP-06 APPARATUS

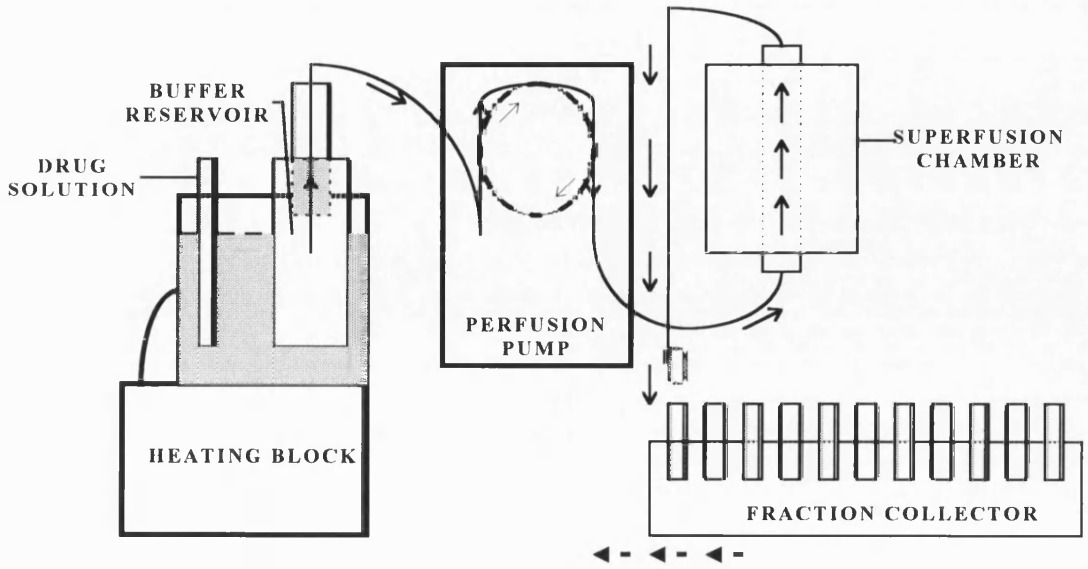
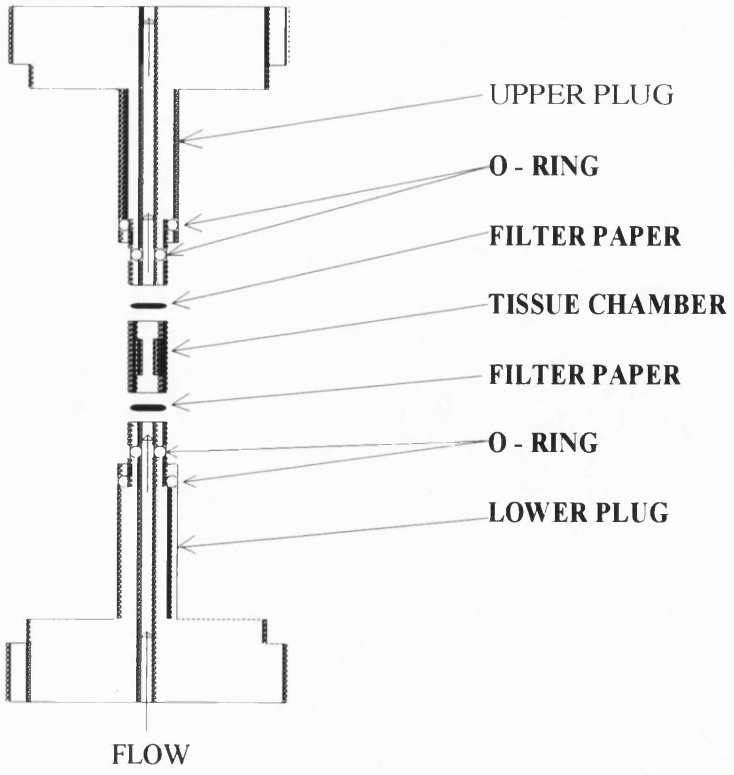


FIGURE 3.1B. DETAILED VIEW OF BRANDELL SUPERFUSION CHAMBER



with cortical and then with hippocampal synaptosomes. The standard superfusion protocol of Thorne *et al.*, (1991) was adapted if an improvement in terms of sensitivity of response, could be demonstrated.

3.2. METHODS

3.2.1. SYNAPTOSOME PREPARATION

Cortical synaptosomes were isolated on Percoll gradients essentially as described by Dunkley *et al.*, (1988). Excised tissue was homogenised (10% w/v in 0.32 M sucrose, pH 7.4) in a pre-cooled glass-Teflon homogeniser of 0.31 mm clearance, by 2 x 6 strokes at 200 rpm. The supernatant (S_1) formed from a 10 min 1000 g spin, was diluted with 0.32 M sucrose and 2 ml aliquots were layered onto the gradients using a large bore needle (19 G) This and all subsequent centrifugation steps were carried out in the Sorvall RC 5B Superspeed centrifuge (SM 24 rotor). Four gradients were used per cortex.

After layering, the Percoll gradients were centrifuged for exactly 5 min (excluding acceleration and deceleration times) at 32,000 g. The synaptosome fraction F4, at the interface of the 23% and 15% Percoll/sucrose layers was removed with a Pasteur pipette and washed in two resuspension/centrifugation steps prior to the final resuspension. In each step the F4 fractions from 2 gradients were pooled, resuspended in 10 ml of buffer and centrifuged for 15 min at 15,000 g. The buffer used throughout was oxygenated Kreb's bicarbonate. After the final wash the pellet was gently resuspended in buffer with a Pasteur pipette, to a protein concentration of approximately 1 mg/ml. All centrifugation steps were carried out at 4°C and tissue preparations were held on ice until use, to preserve synaptosomal viability.

3.2.2. CHOLINE UPTAKE

The F4 synaptosome preparation (950 µl) was pre-incubated at 37°C for 10 min before the addition of [^{3}H]choline (100 µl: 16 µM, 0.5 mCi/ml: prepared by 1:1 dilution of stock [^{3}H]Ch with 20 µM choline chloride; final assay concentration 0.8 µM). After 30 min at 37°C the mixture was centrifuged in a bench microfuge (1 min, 3000 g). The supernatant was discarded, removing excess [^{3}H]Ch that had not been taken up by the synaptosomes. The pellet was gently resuspended in Kreb's buffer to 1 ml; triplicate samples (10 µl) were filtered on Whatmann GFC filters pre-moistened with Kreb's buffer in a Millipore manifold

filtration unit under gentle suction and immediately washed with 5 ml Kreb's buffer to determine [^{3}H]Ch uptake. Radioactivity was measured in 3 ml of scintillant.

3.2.3. SUPERFUSION

The superfusion parameters: flow rate; mg protein per chamber, size of agonist pulse and size of air bubble, were initially derived from Moss & Wonnacott (1985), as modified by Rapier *et al.* (1988), and Thorne (1990).

Routinely, all six superfusion chambers of the SP-06 apparatus were used. In each case, the synaptosome sample was placed on a Whatmann GFF filter disc, pre-moistened with Kreb's buffer, in the superfusion chamber (see figure **3.1B.**). Another pre-moistened Whatmann GFF filter disc was positioned above the synaptosomes before the lower and upper plugs were gently positioned in the superfusion chamber.

Briefly, 150 µl of the Percoll F4 fraction (approximately 200 µg protein) were loaded per chamber and superfused with Kreb's buffer (oxygenated for 2 hours before the start of the superfusion and adjusted to pH 7.4 with NaOH if necessary) at a flow rate of 250 µl/min. The superfusion chamber was positioned over the fraction collector which was set to collect superfusate fractions at three minute (750 µl) intervals, directly into minivials. To each fraction 3 ml of scintillant was added before the radioactivity was measured.

After a 45 min washout period, the synaptosomes were stimulated at 30 min intervals with a 20 s (83 µl) pulse of agonist (made up in Kreb's buffer) separated from the bulk flow of buffer by 10 s air bubbles to prevent diffusion of the agonist along the length of the superfusion tubing, ensuring that the drug reached the chamber as a discrete pulse. One or two such stimulations were routinely administered before a final stimulation with 20 mM KCl was given.

3.2.4. EXPRESSION OF RELEASE DATA

The responses to successive stimulations were designated S1, S2 and S3 and evoked release was expressed in three different ways:

i) Fmol [^{3}H]ACh Released/mg Protein.

In all experiments, the stimulus-evoked release of [^{3}H]ACh was determined as the amount of radioactivity above baseline for three fractions after the stimulus . The baseline due to basal release, was determined by first-order extrapolation of the amount of radioactivity released during the three fractions immediately prior to the stimulus (Rowell *et al.*, 1987). Radioactivity was converted to femtomoles of ACh by reference to the specific radioactivity of [^{3}H]Ch used to load the synaptosomes. This method of expressing the data is the most widely used in this thesis, and was adopted because it provides an comparison of evoked release in absolute terms, after Rapier *et al.*, (1988). The only drawback of this method is that the stimulus-evoked release was proportional to the uptake of the labelled transmitter. Quite simply, if the uptake of transmitter was relatively low then the resultant baseline release was also relatively low and stimulus-evoked [^{3}H]ACh release was found to be proportionally lowered. This becomes a problem if the results of two experiments with quite different [^{3}H]Ch uptake values are compared. This problem was countered by introducing a correction factor into the calculation of the evoked [^{3}H]ACh release, such that all values expressed were corrected for a [^{3}H]Ch uptake value of 80 pmol/mg hippocampal protein, or 40 pmol/mg cortical protein, see section **3.3.3.**

ii) Percentage Increase Over Baseline.

As above, the rate of baseline release during the three fractions immediately prior to the stimulus was calculated by a first-order elimination equation (Rowell & Hillebrand, 1994), the resulting correlation coefficients being greater than 0.95 in all experiments. Baseline release was then extrapolated under the stimulus-induced peaks, and the increase in [^{3}H]ACh release was determined as the area under the curve expressed as the percentage of baseline, following the methods of Rowell & Winkler (1984) as modified by Rowell & Wonnacott (1990). The major merit of this method is that no correction factors need to be introduced into the calculation since evoked release is being expressed as a proportion of the baseline, so inherent variations of this baseline are integral to the calculation.

iii) % Radioactivity Remaining.

Again, the stimulus-evoked release was calculated by extrapolating the baseline under the peaks and the increase in [^{3}H]ACh release was determined as the area under the curve expressed as the percentage of the radioactivity present in the tissue at the time of the

stimulation (by summing all subsequent fractions including the radioactivity remaining on the Whatmann GFF filters at the end of the superfusion experiment). This method of expressing release data has been particularly used for short superfusion experiments such as the ten minute $^{86}Rb^{+}$ flux assays of Marks *et al.*, (1993), and Grady *et al.*, (1992), but is less useful for a one or two hour superfusion study because the calculation of radioactivity remaining becomes laborious. In this method no correction factors need to be introduced since the variability in [^{3}H]Ch uptake is reflected in the amount of radioactivity remaining on the filters at the end of the experiment. This method is dependent on a homogenous synaptosome preparation since any experimental variation in distribution of the [^{3}H]Ch-loaded synaptosomes into the chambers will be emphasised by differences in the radioactivity remaining on the filters: though not widely quoted in this thesis, this method of expressing the superfusion release data is included for completeness and to emphasise the reproducibility of the superfusion protocol.

3.3. RESULTS AND DISCUSSION

3.3.1. CHOLINE UPTAKE

A prerequisite for a sensitive superfusion system is a means of radiolabelling the neurotransmitter for its identification. Here synaptosomes were loaded with [^{3}H]Ch which is rapidly converted to [^{3}H]ACh (Wonnacott & Marchbanks, 1976), based on previously established conditions (Thorne, 1990) see section **3.2.2.**

Cortical synaptosomes typically accumulated **45.7** ± **5.0** pmol/mg protein/30 min (mean ± SEM; n=8), compared with **99.8** ± **8.3** pmol/mg protein/30 min (mean ± SEM; n=24), for hippocampal synaptosomes. The lower uptake of the cortical synaptosomes is likely to reflect the lower proportion of cholinergic terminals in this tissue. In a study involving the measurement of [^{3}H]Ch uptake into human cortical and hippocampal synaptosomes, Rylett *et al.*, (1983) reported that sucrose-gradient isolated hippocampal synaptosomes accumulated 0.505 pmol Ch/LDH unit/min compared to a figure of 0.232 pmol Ch/LDH unit/min for cortical synaptosomes. The authors chose to express the uptake in these units because for the more crude synaptosome fraction obtained from sucrose gradients, a useful measure of synaptosomal integrity is the level of occluded LDH activity. While it is not possible to directly compare these figures with those obtained in this study, it is clear that the cortical synaptosomes accumulate approximately half as much [^{3}H]Ch as the hippocampal synaptosomes, a similar proportion to that reported here. Other studies

have reported a range of uptake values for cortical and hippocampal synaptosomes: Raiteri *et al.*, (1984) using Ficoll-sucrose-isolated cortical synaptosomes and a rather different incubation method to determine uptake, reported 80.5 ± 17.2 pmol/mg protein. Thorne *et al.*, (1988) using essentially the procedure described in section **3.2.2.**, reported an uptake of 145 pmol/mg for Percoll-isolated hippocampal synaptosomes, compared with 22 pmol/mg protein for sucrose-isolated synaptosomes from the same brain region; an uptake range of 100-150 pmol/mg protein, over a period of several months for Percoll-isolated hippocampal synaptosomes has been reported by the same author (Thorne, 1990); and in 1991 Thorne *et al.*, published an uptake value of 160 pmol/mg protein for the same preparation of hippocampal synaptosomes.

In common with the literature discussed above, the uptake of [^{3}H]Ch showed some variability between experiments in the present study (the range for hippocampal synaptosomes was 40-120 pmol/mg protein/30 min). Factors contributing to this variability probably include fluctuations in incubation temperature as well as the age of the [^{3}H]Ch and cold choline stocks but ultimately the major variable was the quality of the synaptosome preparation. The uptake of [^{3}H]Ch was routinely used as a diagnostic tool to determine the viability of the synaptosome preparation. Consequently, uptake was taken into consideration in analysing the subsequent release data, as the release of radiolabel was proportional to uptake.

To directly compare stimulus-evoked release data, expressed as fmol/mg protein, the data were normalised to an uptake of 80 pmol/mg protein/30 min. The figure represents the mean for [^{3}H]Ch uptake into hippocampal synaptosomes after the first few months of the project. As the protocol became more familiar, the uptake value increased but it is important to note that the value that the uptake is corrected to is not important *per se*, rather it is important that the correction is made.

For example, 1 μM nicotine was often used as a control in experiments investigating the phenomenon of nicotinic agonist-evoked release: an examination of the [^{3}H]ACh release evoked by 1 μM nicotine in uncorrected fmol/mg protein, compared with the [^{3}H]Ch uptake value for each respective experiment, is instructive:

	Release evoked by 1 μM Nicotine (fmol/mg protein)	[^{3}H]Ch Uptake (pmol/mg/30 min)	Corrected release (fmol/mg protein)
Experiment 1	897	62.5	1148
Experiment 2	1137	84.8	1205
Experiment 3	1646	105	1254

Examination of the uncorrected data is not encouraging since it would appear that the variation in response to the 1 μM nicotine stimulus was very great. However when the [^{3}H]Ch uptake values for the individual experiments were taken into consideration and the release data corrected for an uptake of 80 pmol/mg protein/30 min, the responses to nicotine were very similar, suggesting that the correction is useful.

In summary, the uptake values described in this chapter for both hippocampal and cortical synaptosomes are typical for the literature, and it is interesting to note that in the work of Thorne *et al.*, (1988; 1990; 1991) there was a significant variation in uptake over a period of time, just as in the present study. Having established that the Percoll procedure produced viable synaptosomes (tested by uptake of [^{3}H]Ch), it was important to verify that the synaptosomes would respond to a general depolarising agent, such as KCl.

3.3.2. RESPONSES TO VARYING CONCENTRATIONS OF POTASSIUM

Pilot studies were carried out with cortical synaptosomes to determine if the potassium was efficacious as a depolarising agent and if so, how the transmitter release varied with KCl concentration.

For the purposes of clarity, concentrations of KCl are expressed in millimolar, above that concentration normally found in Kreb's buffer (2.8 mM), thus Kreb's buffer is defined as 0 mM KCl. A 20 mM KCl pulse was used in each chamber as the first stimulus, S1, in order to standardise the response of the synaptosomes. For S2 the KCl concentration was varied between 0 mM (Kreb's buffer) and 50 mM. A pulse of Kreb's buffer was used as S3 in all six chambers. The S1 response was 198 ± 23 fmol [^{3}H]ACh released/mg protein (mean ± SEM). The standard error indicating the fairly low variability between chambers in the Brandell SP-06 apparatus. The responses to the S2 stimulus are represented in figure **3.2.**

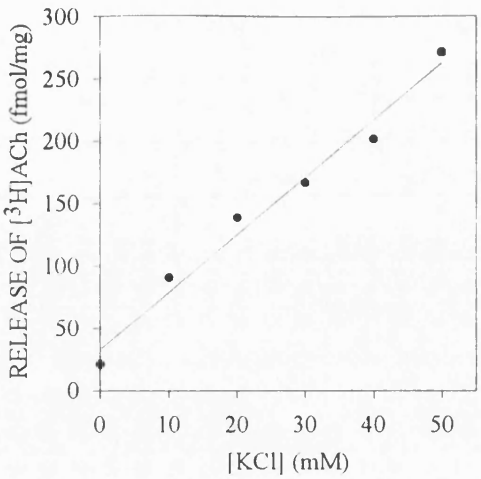


FIGURE 3.2. S2 KCl-EVOKED RELEASE OF [^{3}H]ACh. Synaptosomes were loaded with [^{3}H]Ch and superfused. After a 45 min washout period, all chambers were stimulated with a 20 s pulse of 20 mM KCl, S1, in Kreb's bicarbonate buffer. This was followed 30 min later by a second 20 s pulse of KCl (S2, 0-50 mM) shown above.

The S3 buffer pulse as well as the 0 mM (buffer) S2 stimulus, attempted to quantify the 'air bubble effect' reported by Thorne (1990), who reported that the air bubble separating test drug from buffer, itself caused an apparent release of [^{3}H]ACh of up to 30% of maximal agonist-evoked release, emphasising how sensitive the synaptosomes are to mechano- as well as chemical stimulation. The 'air bubble effect' amounted to 10.4 ± 5.1 fmol [^{3}H]ACh released/mg protein (mean ± SEM), representing 6% of the standard (20 mM) KCl-evoked release, and <1% of maximal nicotinic agonist-evoked release (see chapter 4).

Expressed graphically, the release of [^{3}H]ACh increases in direct proportion to increased KCl concentration, over the concentration range tested. A variety of KCl concentrations are found in the superfusion literature regarding KCl-evoked [^{3}H]ACh or [^{3}H]dopamine release: Rapier *et al.*, (1988; 16 mM and 28 mM), Thorne (1990; 20 mM), Grady *et al.*, (1992; 20 mM), and Rowell & Hillebrand (1994; 15 mM). Since the evoked release was apparently linear, at least up to a concentration of 50 mM KCl, in order to facilitate comparison with the previous studies by Thorne (1990), 20 mM KCl was adopted as the standard concentration and used henceforth.

3.3.3. VARIATION IN PROTEIN CONCENTRATION

The ideal situation for studying transmitter release from synaptosomes would be the rapid superfusion of a synaptosomal monolayer. The rapid flow of buffer would ensure that

released transmitter was removed from the vicinity of the synaptosomal receptors, ensuring that any problems of transmitter re-uptake, or positive or negative feedback on the presynaptic receptors, would be minimised. Similarly, a monolayer is ideal because if synaptosomes are packed tightly on top of one another, transmitter release may be masked as synaptosomes take up radiolabelled choline (derived from the hydrolysis of released [^{3}H]ACh) from neighbouring synaptosomes before the superfusate has left the superfusion chamber.

The design of the superfusion chamber in the Brandell apparatus (see figure **3.1.**), particularly the fact that the Kreb's buffer is forced to flow from the bottom to the top of the chamber, ensures that whatever the synaptosomal concentration, the synaptosomes are not crowded onto the lower GFC filter.

In order to optimise the response of [^{3}H]ACh, responses to agonist were examined with varying protein concentrations loaded into the superfusion chamber. Using 10 μM nicotine as the agonist challenge for the S1 and S2 pulses, with 20 mM KCl as the S3 pulse, the protein concentration was varied between approximately 100 μg and 250 μg per chamber. The results are summarised in table **3.1.** below:

PROTEIN CONCENTRATION (mg/chamber)	**94.8 ± 2.6**	**158 ± 4.4**	**237 ± 6.4**
[^{3}H]ACh RELEASE (fmol/mg protein):			
S1: 10 μM NICOTINE	169 ± 0.9	123 ± 13	181 ± 3.7
S2: 10 μM NICOTINE	137 ± 33	109 ± 36	127 ± 26
S3: 20 mM KCl	131 ± 25	105 ± 13	118 ± 2.5

TABLE 3.1: VARIATION IN [^{3}H]ACh RELEASE FROM CORTICAL SYNAPTOSOMES, WITH RESPECT TO DIFFERENT PROTEIN CONCENTRATIONS. Values are fmol ACh released/mg protein (mean ± SEM), corrected for a [^{3}H]Ch uptake of 40 pmol/mg protein/30 min. (n=3).

The experiment was repeated with hippocampal synaptosomes, varying the protein concentration between approximately 80 μg and 200 μg., with 10 μM nicotine again being used for the S1 and S2 pulses, and 20 mM KCl used for S3. The results are shown in table **3.2.** below:

PROTEIN CONCENTRATION (mg/chamber)	197 ± 3.5	132 ± 2.5	84.6 ± 7.4
[^{3}H]ACh RELEASE (fmol/mg protein):			
S1: 10 μM NICOTINE	149 ± 44	210 ± 76	734 ± 91*
S2: 10 μM NICOTINE	109 ± 14	112 ± 30	432 ± 88*
S3: 20 mM KCl	283 ± 11	241 ± 5.7	274 ± 35

TABLE 3.2. VARIATION IN [^{3}H]ACh RELEASE FROM HIPPOCAMPAL SYNAPTOSOMES, WITH RESPECT TO DIFFERENT PROTEIN CONCENTRATIONS. Values are fmol ACh released/mg protein (mean ± SEM), corrected for a [^{3}H]Ch uptake of 80 pmol/mg protein/30 min. (n=3). Significantly different from other protein concentrations, *$p<0.05$.

Varying the concentration of cortical synaptosomes loaded into the superfusion chamber seemed to have little effect upon the sensitivity to either nicotine or potassium, with none of the protein concentrations tested producing significantly different responses when compared to either of the other protein concentrations. [1]

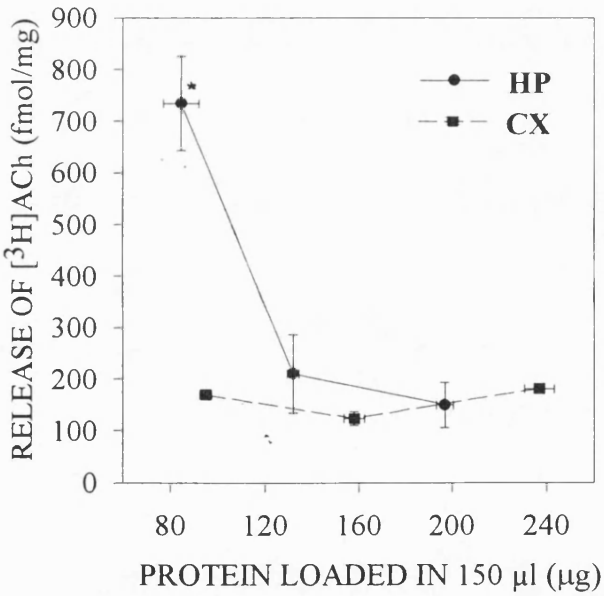


FIGURE 3.3. THE EFFECTS OF VARYING PROTEIN CONCENTRATION IN THE SUPERFUSION CHAMBER. [^{3}H]ACh release was examined by stimulating three different concentrations of synaptosomes, in duplicate chambers, for hippocampal (HP) and cortex (CX) preparations. The S1 stimulation with 10 μM nicotine is shown. Symbols represent the mean ± SEM of three independent determinations for each tissue. The release reported for the lowest concentration of HP synaptosomes is significantly different (*) from both other concentrations at the $p<0.05$ level.

[1] ($p<0.05$, using the standard test (Parker's adaptation of Student's t-test) for the differences between the means of 2 small samples, population variances not assumed to be equal)

In contrast, the lowest concentration of hippocampal synaptosomes yielded significantly higher [1] responses to 10 μM nicotine in both the S1 and S2 stimulations. The data for S1 stimulation of hippocampal and cortical synaptosomes is summarised in figure **3.3.** above. However, KCl-evoked release was consistent at all three protein concentrations examined in both brain regions.

The rise in evoked release is not directly proportional to the fall in protein concentration, so clearly there is no simple explanation for these results. If the difference in responsivity with the hippocampal synaptosomes were simply a result of crowding/re-uptake of transmitter, similar differences in response to S3 (20 mM KCl) would be expected. The absence of such increases suggests that while re-uptake may complicate the observed transmitter release at higher protein concentrations, it is not the only factor involved. The magnitude of release for the two higher protein concentrations of synaptosomes mirror that obtained by Thorne (1990), using similar loading conditions. High concentrations of hippocampal synaptosomes in the superfusion chamber, nevertheless caused a significant drop in transmitter release which is undesirable: the increased response to nicotine at the lowest protein concentration represents a step forward in the development of a more sensitive superfusion assay, and as such this concentration of protein (between 80 and 100 μg/chamber) was adopted as the standard protocol for future hippocampal superfusion studies.

For both cortical and hippocampal nicotine-evoked [^{3}H]ACh release, the S1 agonist challenge provokes more transmitter release than the S2 challenge. For the cortical synaptosomes the S2/S1 ratio for 1 μM nicotine-evoked release varied between 0.89 and 0.70, depending on the concentration of protein in the superfusion chamber. In comparison, the S2/S1 ratio for hippocampal synaptosomes varied between 0.73 and 0.53, i.e. at the higher protein concentrations, the S2 response was approximately half the magnitude of the S1 response. The reasons for this attenuation of agonist-evoked response upon repetitive agonist stimulation include pool depletion and nAChR desensitisation, and are discussed in section **5.3.1.**

Hippocampal synaptosomes release more [^{3}H]ACh in response to both nicotine and KCl than cortical synaptosomes. One reason for the apparent difference is that the number of cholinergic terminals in the cortex as a proportion of the total number of nerve terminals, may be smaller than in the hippocampus. If a smaller proportion of the F4 cortical synaptosomes are cholinergic, the average [^{3}H]Ch uptake values will be lower than in the

hippocampus, which is exactly what was recorded (cortex: 45.7 pmol/mg protein/30 min; hippocampus: 99.8 pmol/mg protein/30 min). This is important since released [^{3}H]ACh was corrected for [^{3}H]Ch uptake. Taking such differences in [^{3}H]Ch uptake into account, the KCl-evoked release in both tissues was very similar, as were the responses to nicotine at the two highest hippocampal protein loading concentrations. At the lowest hippocampal protein concentration, the responses to 10 μM nicotine were still significantly higher than the corresponding cortical responses, even taking into account the differences in [^{3}H]Ch uptake.

3.3.4. TYPICAL SUPERFUSION PARAMETERS FOR HIPPOCAMPAL SYNAPTOSOMES

Having established the loading conditions for the chamber and the typical uptake of the hippocampal synaptosomes (section **3.3.1.**), it was necessary to quantify the superfusion profile. The baseline in a typical superfusion profile varies in direct proportion to the [^{3}H]Ch uptake, high uptake generally produces a higher baseline and vice versa. Nevertheless it was possible to quantify this baseline by examining the basal release of transmitter at selected points during the experiment. Fractions 13, 23 and 33 represent points just prior to the S1, S2 and S3 stimulations, respectively. Mean released radioactivity values (cpm), for the hippocampal superfusions were (mean ± SEM; n=25);

Fraction 13: 9271 ± 832
Fraction 23: 8208 ± 704
Fraction 33: 7149 ± 561

Radioactivity released was assumed to be [^{3}H]ACh, since previous studies (Rowell & Winkler, 1984; Thorne 1990) have shown that in excess of 90% of the radioactivity was [^{3}H]ACh. The shallow slope of the baseline (see figure **3.4.** for a typical example), demonstrates that the synaptosomes release transmitter slowly and steadily. It is worth stressing the important features of this profile, including: the stability of the baseline, making the extrapolation under the peaks straightforward; (see section **3.2.4.**) and the relative height of the peaks demonstrating assay sensitivity. A sharp baseline slope is indicative of a poor synaptosomal preparation, and assays involving such 'leaky' synaptosomes were discarded.

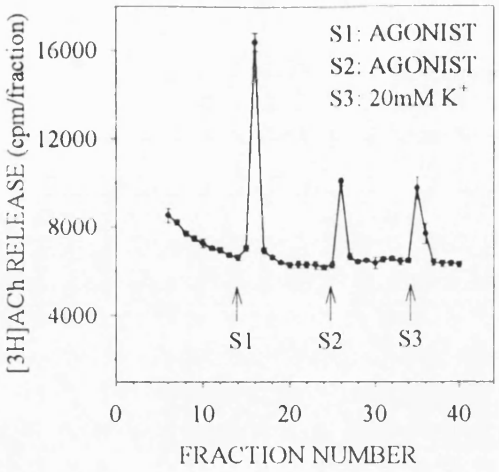


FIGURE 3.4. TYPICAL SUPERFUSION PROFILE. Synaptosomes were loaded with [^{3}H]choline and superfused. After a 45 min washout period, the synaptosomes were stimulated with a 20 s pulse of 5 μM nicotine in Kreb's bicarbonate buffer: S1. This was followed 30 min later by a second 20 s pulse of 5 μM nicotine: S2. Thirty minutes later, a 20 s pulse of 20 mM KCl was administered: S3. Values are the mean ± SEM from one experiment, n = 6. ↑ Indicates pulse arriving at the synaptosomes.

Generally, the evoked release for all agonists studied, and potassium, followed a similar pattern. The peaks were generally sharp with the majority of the evoked [^{3}H]ACh contained in one, or more rarely two fractions.

3.3.5. THE CALCIUM-DEPENDENCE OF [^{3}H]ACh RELEASE

To investigate whether the superfusion system was monitoring a physiological response, various experiments were carried out using Kreb's buffer lacking Ca^{2+} (buffer osmotically corrected with Na^+). Table **3.3.** shows the calcium dependence of a number of superfusion parameters, for a series of experiments using 20 mM KCl as the stimulus.

	KREB'S + Ca^{2+}	KREB'S - Ca^{2+}	Ca^{2+} DEPENDENCE
% fmol/mg	100 ± 16	57.7 ± 8.6	42.3% *
% BASELINE	125 ± 18	55.2 ± 6.2	55.6% *
% RADIOACTIVITY REMAINING	3.44 ± 0.77	1.73 ± 0.22	49.7% *

TABLE 3.3: THE Ca^{2+}-DEPENDENCE OF KCl-EVOKED [^{3}H]ACh RELEASE Values are % fmol [^{3}H]ACh released/mg protein, corrected for a [^{3}H]Ch uptake of 80 pmol/mg protein/30 min (assuming 100%, release in presence of Ca^{2+}), total peak area as a percentage of the baseline, and peak area as a percentage of the radioactivity remaining (mean ± SEM; n=6). * = $p<0.05$.

Whether the evoked release was calculated in terms of fmol [^{3}H]ACh released/mg protein, or as a percentage increase over the baseline, or as a percentage of the radioactivity remaining on the filters at the end of the superfusion experiment, the results in the absence of Ca^{2+} are significantly lower than in normal Kreb's buffer, yielding a calcium dependency of approximately 50%.

Another interesting effect of superfusing in the absence of calcium is revealed by an examination of the baseline release. If the release at fraction 23, immediately prior to the S2 pulse is examined for example, it is clear that the basal release differed significantly ($p<0.05$) between normal and Ca^{2+}-free Kreb's, (mean ± SEM; n=5):

Kreb's Fraction 23: 12574 ± 950 cpm

- Ca^{2+} Fraction 23: 16032 ± 966 cpm

The higher baseline and steeper slope of this baseline in the absence of calcium indicated that the synaptosomes were less metabolically stable, releasing the transmitter at a higher basal rate than those synaptosomes superfused with standard Kreb's buffer. The baseline in the presence of calcium was higher than that reported in section **3.3.3.** but this is a non-significant difference and is due to experimental variation and the small number (n=5). Rowell & Winkler (1984) observed that nicotine-evoked release of ACh from mouse cerebral cortical synaptosomes was 58% calcium dependent. The authors postulated that the non-calcium dependent release could be a result of mobilisation of internal Ca^{2+} stores which contribute to the release process (Crosland *et al.*, 1983).

To investigate the possibility that mobilisation of internal Ca^{2+} artificially lowered the calcium dependence, the experiments were repeated in the presence of 5 μM EDTA. An advantage of using a chelating agent such as EDTA was that not only would any internal Ca^{2+} be chelated, but also any contaminating Ca^{2+} in the distilled water used to prepare the superfusion buffers, such external Ca^{2+} contamination might also be a factor in the relatively low calcium dependence The pH of the Kreb's buffer containing EDTA was carefully maintained at pH 7.4, and the results are outlined in table **3.4.**

	KREB'S	KREB'S + EDTA	Ca^{2+} DEPENDENCE
% FMOL/MG	100 ± 5.0	18.8 ± 3.9	81.2% *
% BASELINE	132 ± 12	13.7 ± 2.3	89.6% *
% RADIOACTIVITY REMAINING	3.22 ± 0.14	1.00 ± 0.14	68.9% *

TABLE 3.4: THE EFFECT OF 5 μM EDTA ON EVOKED [^{3}H]ACh RELEASE Values are fmol [^{3}H]ACh released/mg protein, corrected for a [^{3}H]Ch uptake of 80 pmol/mg protein/30 min., total peak area as a percentage of the baseline, and peak area as a percentage of the radioactivity remaining, (mean ± SEM; n=6). * = $p<0.05$.

In the presence of EDTA, the calcium dependence of the KCl-evoked release averaged 79.9%, a figure which compares favourably with the reports of Rowell & Winkler (1984), Thorne *et al.*, (1991), and Rapier *et al.*, (1988), the latter reporting that KCl-evoked [^{3}H]dopamine release from striatal synaptosomes was 63.9% dependent on exogenous calcium. The superfusion baseline in the presence of EDTA was significantly higher ($p<0.05$), with a steeper slope than the baseline with normal Kreb's buffer and a comparison of the basal release at fraction 23, immediately prior to the S2 pulse illustrates this point, (mean ± SEM; n=5):

Kreb's Fraction 23:	8886 ± 775 cpm
+ 5 μM EDTA Fraction 23:	13292 ± 1081 cpm

3.4. SUMMARY OF CHAPTER 3

The aim of this chapter was to establish a working protocol for the measurement of [^{3}H]ACh release from hippocampal synaptosomes using the Brandell SP-06 superfusion apparatus. A large amount of work had already been carried out by Thorne (1990), as far as establishing the optimal conditions for isolating hippocampal synaptosomes using Percoll gradients, and loading these synaptosomes with [^{3}H]Ch. This expertise was adopted, but a new protocol was needed to maximise sensitivity using the automated apparatus. This protocol needed to cover such factors as: the amount of protein loaded per superfusion chamber; the response of the synaptosomes to a general depolarising agent such as KCl; and the calcium-dependence of evoked [^{3}H]ACh release. Taking Thorne's protocol as a starting point, modifications were made if increases in sensitivity could be demonstrated.

As a result of the results presented in this chapter, the following superfusion protocol was adopted. Routinely, Percoll-enriched synaptosomes were prepared from the hippocampi of two rats. These synaptosomes were loaded with [^{3}H]Ch (0.8 μM; 30 min), and approximately 100 μg loaded into each superfusion chamber. The synaptosomes were superfused at 37°C with 250 μl of Kreb's buffer/min, and 3 min fractions collected in minivials. Scintillant (3 ml) was added to the superfusate, the mixture mixed and counted in a Packard 1600 scintillation counter. A routine superfusion experiment consisted of a washout period (45 min) to remove excess [^{3}H]Ch and achieve a stable baseline, followed by stimulations (agonist or KCl) at 30 min intervals. It was found that the single biggest variable in the protocol was the synaptosomal uptake of [^{3}H]Ch, which in turn determined the extent of basal and evoked [^{3}H]ACh release. Evoked release could be expressed in a number of ways: as an absolute number of femtomoles of [^{3}H]ACh released/mg of protein loaded into the superfusion chamber, necessitating correcting for the variable uptake of [^{3}H]Ch; as a percentage increase over the baseline; and as a percentage of the radioactivity remaining on the filters at the end of the superfusion system.

To put the results reported in this chapter, into context with the superfusion literature is quite difficult, not only is the measurement of [^{3}H]ACh release fairly rare, especially compared with [^{3}H]dopamine release, but the differences in superfusion apparatus design, and protocol in different laboratories, make direct comparison between studies difficult. To take an example, Grady *et al.*, (1992; 1994) used an open superfusion system at room temperature, with no dead volume, a pulse size of 200-300 μl and fractions collected at 30 s, or minute intervals to characterise nAChR-evoked [^{3}H]dopamine release from mouse striatum. The present study used a closed superfusion system maintained at 37°C, with a pulse size of 83 μl in a total chamber volume of 200 μl, collected fractions of 750 μl at 3 min intervals with the ultimate aim of characterising nAChR-evoked release from rat hippocampus. Nevertheless, if differences between the differing superfusion systems are taken into account there is be no reason why comparisons should not be instructive.

Having defined a protocol for the sensitive measurement of [^{3}H]ACh release from hippocampal synaptosomes, and determined that the synaptosomes were not only responsive to challenges of KCl, but that such evoked release was calcium-dependent, the next chapter moves on to deal with agonist-evoked release.

4.1 INTRODUCTION

The objective of this part of the study was to use the Brandell SP-06 apparatus to examine nicotinic agonist-evoked [^{3}H]ACh release from superfused, F4 synaptosomes produced by the Percoll method (chapter 2).

A variety of aspects of the agonist-evoked transmitter release were examined in turn. Firstly, the calcium dependence and nicotinic nature of the nicotine-evoked release were verified. Then, satisfied that the evoked release was both calcium dependent and mediated via nAChR, the next stage in the pharmacological characterisation process was to examine the concentration dependence of agonist-evoked release. This was accomplished by testing the efficacy of nicotine, then several other nicotinic agonists, to evoke release of [^{3}H]ACh over a range of concentrations, which varied according to the agonist being studied. By comparing the results obtained with the different agonists, an agonist profile of the hippocampal nAChR was built up, which allowed the delineation of several common features of nicotinic agonist-evoked release. Furthermore the EC_{50} values obtained for agonist-evoked release were compared to agonist pharmacological profiles for the same agonists in two different experimental paradigms for defined nAChR subtypes: mouse M10 fibroblast cells and Xenopus oocytes stably expressing chick $\alpha4\beta2$ nAChR. This pharmacological comparison enables one to speculate on the subtype or subtypes of nAChR expressed presynaptically in the rat hippocampus, and is especially useful in light of the paucity of subtype-specific ligands that could be used to more directly elucidate the identity of the hippocampal autoreceptor under study.

In addition to the agonist-evoked release studies, this chapter also reports the results of studies with the $\alpha7$ subtype-selective nicotinic antagonist methyllycaconitine (MLA) and its effects on nicotine-evoked, as well as basal [^{3}H]ACh release. Also included are studies with the acetylcholine esterase (AChE) inhibitor physostigmine (PHY), which has been reported to produce transient improvements in memory related tasks (Davis *et al.*, 1978; Christie *et al.*, 1981). The exact mode of drug action is unclear however since PHY has been reported to interact directly with the nAChR (Pereira *et al.*, 1993) via a novel agonist binding site. The effects of PHY on basal and nicotine-evoked release were thus

examined to ascertain whether the AChE inhibitor was having a direct effect on the hippocampal nicotinic autoreceptors.

4.2. METHODS

Synaptosome Preparation

Hippocampal synaptosomes were prepared and isolated on four step discontinuous Percoll gradients, as described in chapter 2. The F4 fraction was washed twice with Kreb's buffer (10 ml; 15 min at 15,000 g) to remove the Percoll. The pellet was resuspended in Kreb's buffer, preincubated at 37° for 10 min and then loaded with [^{3}H]Ch (0.8 μM; 30 min at 37°C). Excess [^{3}H]Ch was removed by washing with Kreb's buffer (1 ml) followed by centrifugation (1 min at 3,000 g) before resuspension in Kreb's buffer (1 ml) and samples (150 μl) placed in superfusion chambers (see section **2.1.6.**).

Superfusion

The synaptosomes were superfused with Kreb's buffer for an initial 45 min washout period. The effect of nicotinic agonist concentration on [^{3}H]ACh release was studied by varying the agonist concentration in the initial stimulus - S1. The nicotinic nature of the response was studied by introducing buffer, containing the nicotinic antagonist DHßE (1 μM) 15 min after the S1 pulse. The subsequent S2 (agonist) and S3 (KCl) pulses were with drugs made up in the DHßE buffer, see section **2.2.2**. Other antagonists were studied in the same way.

The effect of PHY on basal release was studied by superfusing one half of the chambers with standard Kreb's buffer and comparing the release of [^{3}H]ACh with that obtained by superfusing with Kreb's buffer containing PHY (1 μM) Effects on nicotine-evoked [^{3}H]ACh release were studied by introducing the drug 15 min after the S1 (nicotine) pulse in the standard antagonist protocol. PHY was also used in the standard agonist protocol to ascertain whether the drug could directly provoke [^{3}H]ACh release via the nAChRs.

4.3. RESULTS

4.3.1. CALCIUM DEPENDENCE

To assess the calcium dependence of the nicotinic agonist-evoked [^{3}H]ACh release from hippocampal synaptosomes, a concentration of 5 mM EDTA was used to chelate the Ca^{2+} ions in the Kreb's buffer. In three independent experiments the ability of 1 μM nicotine to evoke release of [^{3}H]ACh in the presence of 5 mM EDTA, was compared to the control response in the presence of Ca^{2+}:

S1 (1 μM nicotine) + Ca^{2+}: 521 fmol/mg protein.

S1 (1 μM nicotine) - Ca^{2+}: 65 fmol/mg protein. (n=3)

This calcium dependency of 87.5% compares well to the dependency of the KCl-evoked transmitter release in section **3.3.4.**.

4.3.2. NICOTINE-EVOKED ACh RELEASE

4.3.2.1. Nicotine Concentration

The effect of nicotine concentration on [^{3}H]ACh release was studied by varying the agonist concentration in the S1 pulse between 100 nM and 50 μM. Figure **4.1.** shows a representative superfusion profile obtained in the course of determining the dose-dependency of nicotine-evoked release. For representation purposes, the baselines for the individual chambers have been averaged with the agonist-evoked release superimposed upon this averaged baseline. However, it can be seen from the small standard error bars of the baseline that the variance between chambers was small.

Figure **4.2A.** shows the dose response curve for the representative experiment shown in figure **4.1.** For each dose response experiment, the data were converted to percent specific release (figure **4.2B.**), taking the [^{3}H]ACh release evoked by 10 μM nicotine as 100%. By converting each individual experiment to percent specific release and then averaging the results, it was possible to estimate an EC_{50} value for nicotine-evoked [^{3}H]ACh release by curve fitting the non-linear Hill equation (figure **4.4.**).

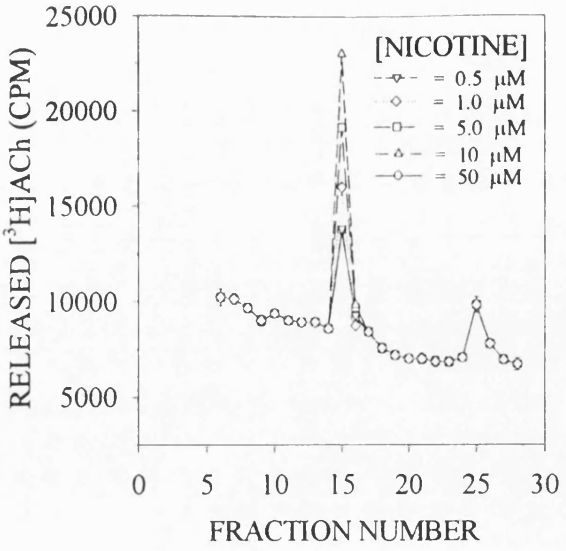
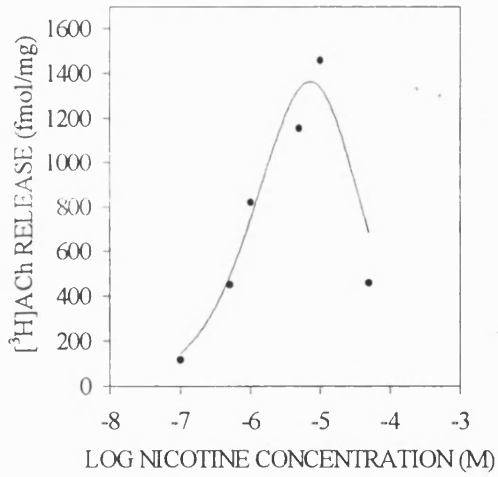


FIGURE 4.1. NICOTINE-EVOKED RELEASE OF [^{3}H]ACh. Synaptosomes were loaded with [^{3}H]Ch and superfused. After a 45 min washout period, the synaptosomes were stimulated with a 20 s pulse of nicotine (S1: 0.5-50 μM) in Kreb's bicarbonate buffer, 30 min later all chambers were stimulated with a 20 s pulse of KCl (S2: 20 mM). Basal release was averaged in the six chambers and evoked release plotted above this common baseline. Basal values are mean ± SEM, n = 6. Each peak represents the data from one chamber, in a representative experiment.

4.2A.



4.2B.

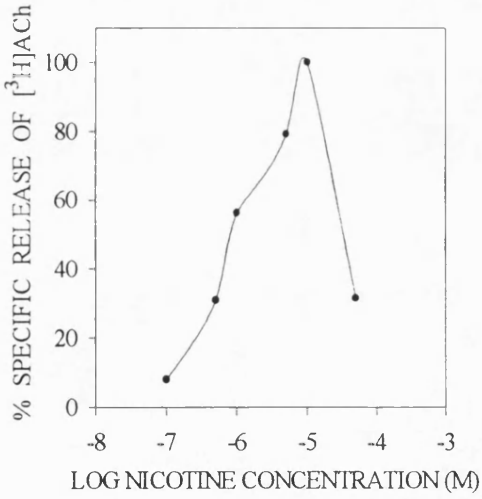


FIGURE 4.2A. NICOTINE DOSE RESPONSE CURVE FOR A REPRESENTATIVE EXPERIMENT. Synaptosomes were loaded with [^{3}H]Ch and superfused. After a 45 min washout period, the synaptosomes were stimulated with a 20 s pulse of nicotine in Kreb's bicarbonate buffer. Basal release was subtracted to give evoked release which was converted to fmol [^{3}H]ACh and plotted against nicotine concentration. Values are from one experiment. The points have been fitted to the non-linear Pliska equation (Pliska, 1994).

FIGURE 4.2B. NICOTINE DOSE RESPONSE CURVE TRANSFORMED TO PERCENT SPECIFIC RELEASE. The data from figure **4.2A.** were converted to percent specific release, taking that release recorded at 10 μM nicotine to be 100%.

Nicotine stimulated a concentration-dependent increase in [^{3}H]ACh release up to 10 μM, after which there was a sharp attenuation in evoked [^{3}H]ACh release. The dose-response curve (figure **4.2A.**) was clearly bell-shaped. The dose-response experiments were repeated and the data from a number of independent experiments are summarised in table **4.1.**

[NICOTINE] μM	fmol ACh RELEASED/ mg PROTEIN	*n*
0.1	122 ± 5	3
0.5	381 ± 62	6
1.0	865 ± 221	6
5.0	904 ± 159	6
10.0	1335 ± 92	6
50.0	689 ± 190	3

TABLE 4.1: THE CONCENTRATION-DEPENDENCE OF NICOTINE-EVOKED [^{3}H]ACh RELEASE. Values are fmol [^{3}H]ACh released/mg protein (mean ± SEM), and were corrected for a [^{3}H]Ch uptake of 80 pmol/mg protein/30 min.

The maximum response to nicotinic stimulation was consistently between four and five-fold higher than the response elicited by a subsequent stimulation with 20 mM KCl:

Maximum response to nicotine (10 μM):

1335 ± 92 fmol/mg protein

Response to 20 mM KCl:

251 ± 59 fmol/mg protein (n=6)

This contrasts sharply with the nicotinic stimulation of [^{3}H]dopamine release from striatal synaptosomes, which is typically about one quarter of that seen with 20 mM KCl (Rapier *et al.*, 1988).

If a dose-response curve for the averaged nicotine-evoked release data is plotted, (figure **4.3.**) the resultant graph is not clearly 'bell-shaped' rather, there a broad 'shoulder' at the nicotine concentrations of 1 μM and 5 μM followed by a further dose-dependent increase in [^{3}H]ACh release. At the highest concentration of 50 μM nicotine there was a sharp attenuation of ACh release.

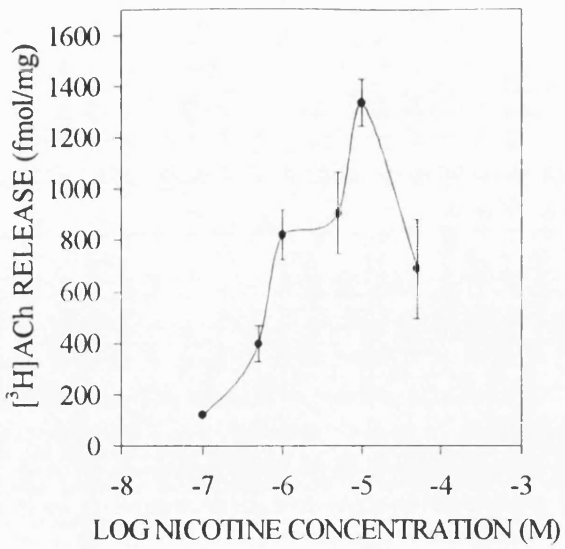


FIGURE 4.3. NICOTINE DOSE-RESPONSE CURVE. Synaptosomes were loaded with [^{3}H]Ch and superfused. After a 45 min washout period, the synaptosomes were stimulated with a 20 s pulse of nicotine in Kreb's bicarbonate buffer. Basal release was subtracted to give evoked release which was converted to fmol [^{3}H]ACh and plotted against nicotine concentration. Values are mean ± SEM, with n = 3-6.

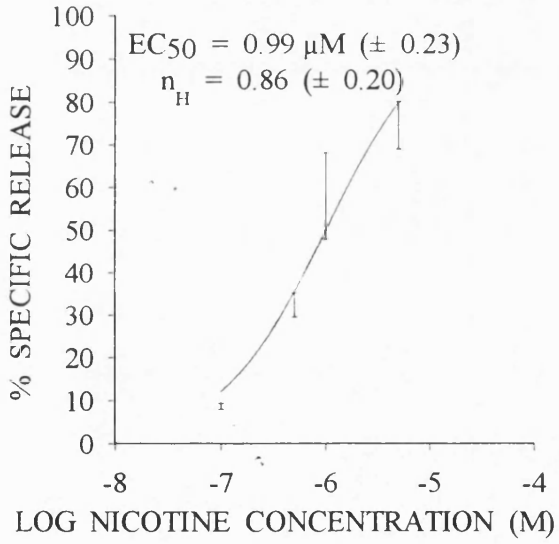


FIGURE 4.4. CURVE FIT TO THE HILL EQUATION FOR NICOTINE. The data from individual nicotine-evoked dose response curves were converted to percent specific release, taking that release recorded at 10 μM nicotine to be 100%, the data averaged, and then fitted to the non-linear Hill equation to enable an estimate of the concentration at which 50% maximal response was observed.

If the release data are fitted to the Hill equation and plotted (figure **4.4.**), it is possible to calculate an EC_{50} value for the nicotine-evoked [^{3}H]ACh release from hippocampal synaptosomes. This value is 0.99 ± 0.23 μM for n=3-6.

4.3.2.2. Nicotinic Nature Of The Response

There exist two possible mechanisms by which nicotine could conceivably evoke [^{3}H]ACh release from the loaded synaptosomes. As a lipophilic molecule it is feasible that the nicotine moiety permeates the synaptosomal membrane directly, and interacts with [^{3}H]ACh-containing vesicles in the active zone of the synaptosome, causing transmitter release. The other possibility is that the nicotine mediates the [^{3}H]ACh release via a presynaptic nAChR.

These two mechanisms can be differentiated by the use of a nicotinic antagonist such as DHßE the principal alkaloid fraction of seeds from *Erythrina spp.* DHßE is one of only a handful of antagonists that potently inhibit the binding of nicotine to membranes from brain, and is equipotent as a neuromuscular and ganglionic blocker (Wonnacott, 1987), suggesting that its potency as an antagonist is not subtype-specific.

Using the standard antagonist protocol (section **2.1.6.2.**) of adding DHßE 15 min before the S2 agonist stimulus and perfusing in the presence of the antagonist for the remainder of the experiment (including the S3 KCl pulse), the ability of 1 µM DHßE to inhibit that release evoked by 10 µM nicotine was tested.

The results are shown in table **4.2.** below:

	CHAMBERS 1-3	CHAMBERS 4-6
S1	10 µM NIC.	10 µM NIC.
FMOL/MG	1269 ± 201	1010 ± 251
% ABOVE BASELINE	76.0 ± 8.0%	75.1 ± 29.5%
S2	10 µM NIC.	10 µM NIC. + 1 µM DHßE
FMOL/MG	456 ± 63.3	127 ± 66.3*
% ABOVE BASELINE	27.8 ± 2.6%	9.1 ± 4.8*
S3:	20 mM KCl	20 mM KCl + 1 µM DHßE
FMOL/MG	203 ± 17.0	214 ± 37.0
% ABOVE BASELINE	12.6 ± 1.3%	15.0 ± 4.3%

TABLE 4.2: ANTAGONISM OF NICOTINE-EVOKED [^{3}H]ACh RELEASE BY DHβE. Fmol/mg values are corrected for a [^{3}H]Ch uptake of 80 pmol/mg protein/30 min. Section **2.1.6.2.** details the administration of the antagonist. Percent above baseline values are calculated by expressing the peak of release over the baseline. *= significantly different from control at the $p<0.05$ level of significance. (n=7)

These data are represented graphically in figure **4.5.**

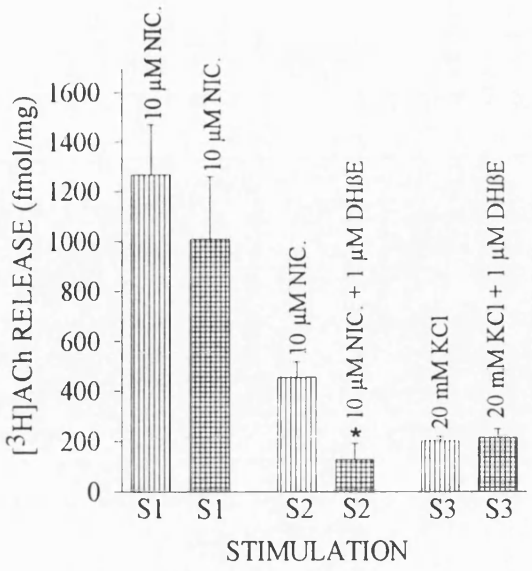


FIGURE 4.5. THE EFFECT OF 1 µM DHβE ON 10 µM NICOTINE-EVOKED [^{3}H]ACh RELEASE. Data were obtained from table **4.2.** Control release defined in the absence of DHβE for S2 and S3. * = significantly different from control at the $p<0.05$ level of significance.

The release of [^{3}H]ACh evoked by 10 µM nicotine was inhibited by 1 µM DHßE. Importantly, the various methods of expressing the data yield the same result. In terms of fmol transmitter released/mg protein, the percent inhibition of the S2 pulse was: 72.1%. This compares with the data expressed as percent increase over the baseline release of transmitter, in which the inhibition of the S2 response was: 67.3%. In both cases there was no inhibition of the potassium-stimulated, S3 response. The preservation of the KCl response is vital to demonstrate that the antagonist is acting specifically at the nAChR and not causing a general depression of transmitter release, indeed by comparing the basal release at fraction 30, 26 minutes after the introduction of the antagonist into the perfusion buffer, it is possible to examine whether basal release of transmitter is significantly altered in the presence of 1 µM DHßE.

Release at F30:

Control: 1243 ± 87 fmol/mg

+ 1 µM DHßE: 1232 ± 69 fmol/mg (n=7)

Plainly, the basal release of transmitter was unaffected by the antagonist. Also evident from the data is the fact that the superfusion system was very reproducible, since the standard error was very small and the difference between the baselines in the control and the antagonist conditions was negligible.

In summary, 1 μM DHßE effects a large antagonism of the nicotine-evoked [^{3}H]ACh release from hippocampal synaptosomes (approximately 90% of the Ca^{2+}-dependent release is antagonised). Thus, it is clear that nicotine is evoking release of tritiated transmitter via presynaptic nAChR.

4.3.3. FURTHER ANTAGONIST STUDIES

Having established that nicotine evoked the release of [^{3}H]ACh via a nAChR-dependent mechanism, the use of further, subtype-specific, nicotinic antagonists could shed light on the contribution of possible nAChR subtypes present on the presynaptic membrane, to the total nicotine-evoked transmitter release.

Using the same antagonist protocol of adding the antagonist to the perfusion buffer after the S1 stimulus and continuously perfusing in the presence of antagonist to the remainder of the experiment, the effects of both 1 μM and 10 μM MLA, on 1 μM nicotine-evoked release, were tested.

	CHAMBERS 1-2	CHAMBERS 3-4	CHAMBERS 5-6
S1	1 μM NIC.	1 μM NIC.	1 μM NIC.
FMOL/MG	873 ± 100	1073 ± 223	1137 ± 234
% ABOVE BASELINE	169 ± 26.0%	182 ± 40.0%	170 ±36.0%
S2	1 μM NIC.	1 μM NIC. +1 μM MLA	1 μM NIC. + 10 μM MLA
FMOL/MG	335 ± 88	286 ± 19.5	181 ± 42*
% ABOVE BASELINE	66.9 ± 19.0%	52.4 ± 4.7%	27.5 ± 7.2%*
S3	20 mM KCl	20 mM KCl +1 μM MLA	20 mM KCl + 10 μM MLA
FMOL/MG	251 ± 59.0	206 ± 23.3	202 ± 51.7
% ABOVE BASELINE	48.2 ± 12.8%	37.7 ± 4.4%	29.9 ± 7.6%

TABLE 4.3: THE EFFECT OF 1 μM AND 10 μM MLA ON THE ABILITY OF 1 μM NICOTINE TO EVOKE TRANSMITTER RELEASE. Fmol/mg values are corrected for a [^{3}H]Ch uptake of 80 pmol/mg protein/30 min. Percent above baseline values are calculated by expressing the peak of release over the baseline at fraction 20 (midpoint of the experiment). * = significantly different from control at the $p<0.05$ level of significance. (n=4)

Both 1 μM MLA and 10 μM MLA were able to inhibit the release of transmitter evoked by 1μM nicotine, but to markedly different extents. If the inhibition of the S2 stimulus is summarised:

Percent inhibition of S2 response:	1 μM MLA	10 μM MLA
Fmol/mg protein	14.6%	46.0% *
% above baseline	21.7%	58.9% *

If the effect on the KCl-evoked [^{3}H]ACh response is examined, it is clear that the MLA was able to inhibit non-nAChR-evoked transmitter release to a small extent.

Percent inhibition of S3:

20 mM KCl response:	1 μM MLA	10 μM MLA
Fmol/mg	17.9%	19.5%

This inhibition, reported previously by Drasdo *et al.*, (1992) was neither statistically significant nor dose dependent.

The possibility that MLA was simply inhibiting basal release of transmitter could be examined by comparing the magnitude of release at fraction 30, an arbitrary point during the superfusion profile that may be used as a marker of basal release:

	Control	+ 1 μM MLA	+ 10 μM MLA
Fmol/mg at F30:	555 ± 44	658 ± 41	679 ± 49
(*n*):	4	7	7

There was no significant inhibition of basal release with either concentration of MLA, suggesting that the alkaloid is inhibiting evoked transmitter release, albeit both nAChR-mediated and to a small extent KCl-evoked release, rather than having more general effects on the overall release profile.

4.3.4. FURTHER AGONIST-EVOKED RELEASE

4.3.4.1. Cytisine

Cytisine is an alkaloid found in the seed of *Laburnum anagyroides*, and was originally characterised as an nicotinic agonist at autonomic ganglia. In binding studies cytisine binds to nAChR with higher affinity than nicotine, low non-specific binding and a slow rate of dissociation from the receptor (Pabreza *et al.*, 1990), making it a useful and widely used tool to study neuronal nicotinic receptors.

The drug was used in the present study over a concentration range identical to that used in the nicotine study, i.e. 100 nM to 50 μM (section **4.3.2.1.**), to study whether the drug could evoke [^{3}H]ACh release from the hippocampal synaptosomes, and if so, how the release profile compared to that obtained with nicotine. The results are shown in table **4.4.**

[CYTISINE] μM	fmol ACh RELEASED /mg PROTEIN
0.1	160 ± 60
0.5	394 ± 124
1.0	770 ± 130
5.0	1181 ± 71
10.0	1456 ± 215
50.0	624 ± 98

TABLE 4.4: THE CONCENTRATION-DEPENDENCE OF CYTISINE-EVOKED [^{3}H]ACh RELEASE. Values are expressed as fmol ACh released/mg protein (mean ± SEM), and were corrected for a [^{3}H]Ch uptake of 80 pmol/mg protein/30 min. (*N*=3).

Expressed graphically (figure **4.6.**) this data yields a dose response curve similar to that obtained with nicotine. From 100 nM to 10 μM, cytisine evokes [^{3}H]ACh release in a concentration-dependent manner. There follows a sharp attenuation in the evoked release at the highest cytisine concentration, again suggesting nAChR desensitisation.

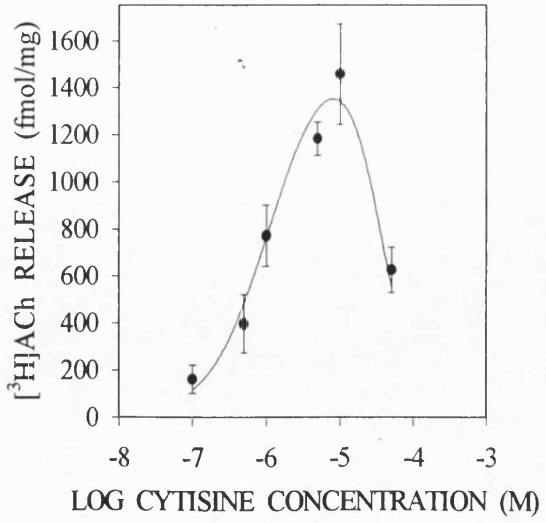


FIGURE 4.6. CYTISINE DOSE-RESPONSE CURVE. Synaptosomes were loaded with [^{3}H]Ch and superfused. After a 45 min washout period, the synaptosomes were stimulated with a 20 s pulse of cytisine in Kreb's bicarbonate buffer. Basal release was subtracted to give evoked release which was converted to fmol [^{3}H]ACh and plotted against cytisine concentration. Values are the mean ± SEM, n = 3. The points have been fitted to the non-linear Pliska equation (Pliska, 1994).

The release data were fitted to the Hill equation enabling an EC_{50} value for cytisine-evoked release to be calculated (figure **4.7.**). This value of 1.06 μM was not significantly different (at the $p<0.05$ level) from that value calculated for nicotine of 0.99 μM (figure **4.4.**).

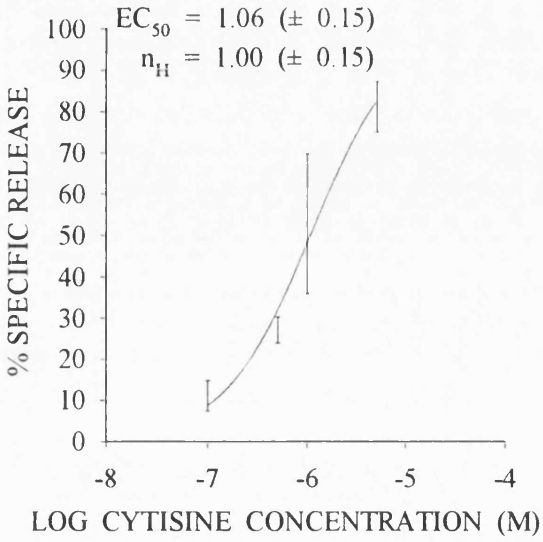


FIGURE 4.7. CURVE FIT TO THE HILL EQUATION FOR CYTISINE. The data from table **4.4.** were converted to percent specific release, taking that release recorded at 10 μM cytisine to be 100%, and then fitted to the non-linear Hill equation to enable an estimate of the concentration at which 50% maximal response was observed.

4.3.4.2. (+)-Anatoxin-A

(+)-Anatoxin-a (ANTX) is a potent nicotinic agonist isolated from blue green algae (*Anabaena flos-aquae*) that can cause deaths of cattle and wildfowl (Carmichael *et al.*, 1975). ANTX is a potent competitor of [^{3}H]nicotine and [^{125}I]-α-Bgt binding to brain membranes (MacAllan *et al.*, 1988; Wonnacott *et al.*, 1991), but such binding assays provide little indication of functional efficacy. ANTX has been used as an effective nicotinic agonist in preparations of cultured neurons (Aracava *et al.*, 1987; Alkondon & Albuquerque, 1990), and has been used to probe central nAChR in both *in vitro* assays (Swanson *et al.*, 1986; 1991; Wonnacott *et al.*, 1991; Thomas *et al.*, 1993) and in various behavioural paradigms (Stolerman *et al.*, 1992).

In keeping with its greater potency in binding studies, ANTX was tested over a different range of concentrations than cytisine and nicotine: from 10 nM to 10 μM. The results are summarised in table **4.5.** represented graphically in figure **4.8.**

[ANTX] (μM)	fmol ACh RELEASED /mg PROTEIN
0.01	14 ± 12
0.1	63 ± 15
0.5	1225 ± 177
1.0	801 ± 118
10.0	179 ± 77

TABLE 4.5: THE CONCENTRATION-DEPENDENCE OF ANTX-EVOKED [^{3}H]ACh RELEASE. Values are expressed as fmol ACh released/mg protein (mean ± SEM), and were corrected for a [^{3}H]Ch uptake of 80 pmol/mg protein/30 min. (n=3).

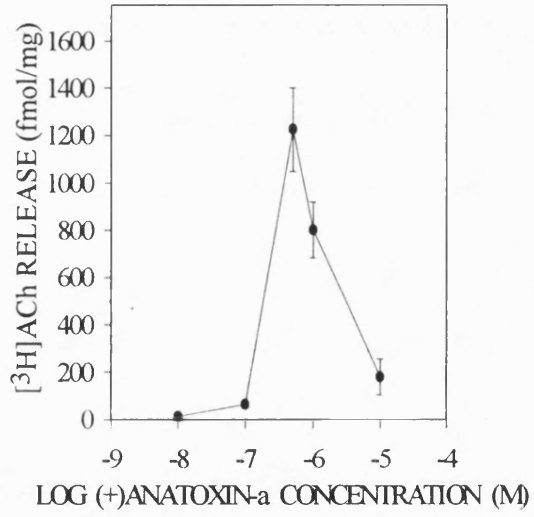


FIGURE 4.8. (+)ANATOXIN-a DOSE-RESPONSE CURVE. Synaptosomes were loaded with [^{3}H]Ch and superfused. After a 45 min washout period, the synaptosomes were stimulated with a 20 s pulse of ANTX in Kreb's bicarbonate buffer. Basal release was subtracted to give evoked release which was converted to fmol [^{3}H]ACh and plotted against ANTX concentration. Values are mean ± SEM, n = 3.

This dose response curve shows the potency of ANTX in the hippocampal synaptosome preparation. The exceptionally steep shape of the graph precludes fitting the data to the Hill equation to estimate the EC_{50}, since to successfully fit the equation without a large inherent error, there must be at least 3 points between 10% and 90% specific release. Since this is not possible with ANTX, a crude EC_{50} was calculated by constructing a linear regression between the concentrations 100 nM and 500 nM (maximal release). The value corresponding to 50% maximal release was calculated and used as the estimate for

the EC_{50}. These individual values were then averaged to give an estimate of the EC_{50} of 0.14 ± 0.04 μM (*n*=3).

4.3.4.3. Iso-arecolone

Iso-arecolone was introduced into neuropharmacological research following the discovery of its high potency as a nicotinic agonist in peripheral pharmacological preparations (Haglid 1967), and has since been used in a variety of *in vivo* behavioural paradigms, in addition to *in vitro* binding studies (Reavill *et al.*, 1987; Reavill *et al.*, 1990), which have suggested that as a competitor at the high affinity [^{3}H]nicotine binding site, the agonist is between 10 and 50 times less potent than nicotine or cytisine. As such, to ascertain whether iso-arecolone was effective at evoking [^{3}H]ACh release from the hippocampal synaptosome preparation, a range of between 10 μM and 500 μM was used in the superfusion assay. The data are summarised in table **4.6.** and represented graphically in figure **4.9.**

[ISO-ARECOLONE] (μM)	fmol ACh RELEASED /mg PROTEIN
10	113 ± 36
25	536 ± 106
50	763 ± 109
75	1546 ± 371
100	1260 ± 330
500	265 ± 51

TABLE 4.6: THE CONCENTRATION-DEPENDENCE OF ISO-ARECOLONE-EVOKED [^{3}H]ACh RELEASE. Values are expressed as fmol ACh released/mg protein (mean ± SEM), and were corrected for a [^{3}H]Ch uptake of 80 pmol/mg protein/30 min. (*N*=3).

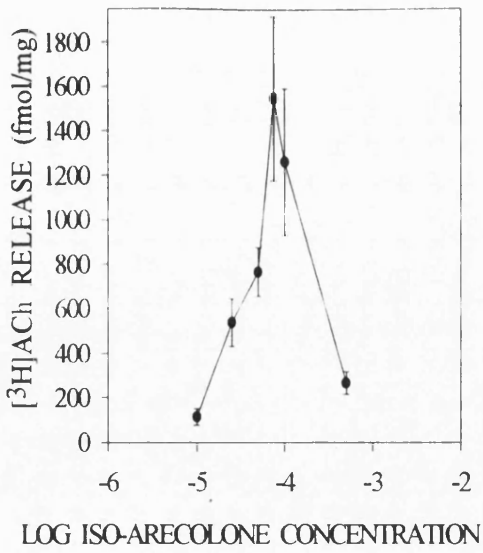


FIGURE 4.9. ISO-ARECOLONE DOSE-RESPONSE CURVE. Synaptosomes were loaded with [^{3}H]Ch and superfused. After a 45 min washout period, the synaptosomes were stimulated with a 20 s pulse of iso-arecolone in Kreb's bicarbonate buffer. Basal release was subtracted to give evoked release which was converted to fmol [^{3}H]ACh and plotted against iso-arecolone concentration. Values are mean ± SEM, n = 3.

In common with the data for ANTX, the dose response curve for iso-arecolone showed a sharp increase in evoked release up to a distinct peak, at 75 μM iso-arecolone, followed by an equally sharp attenuation of evoked release. Fitting the data to the Hill equation (figure **4.10.**) yields an EC_{50} value of 43.2 ± 7.0 μM.

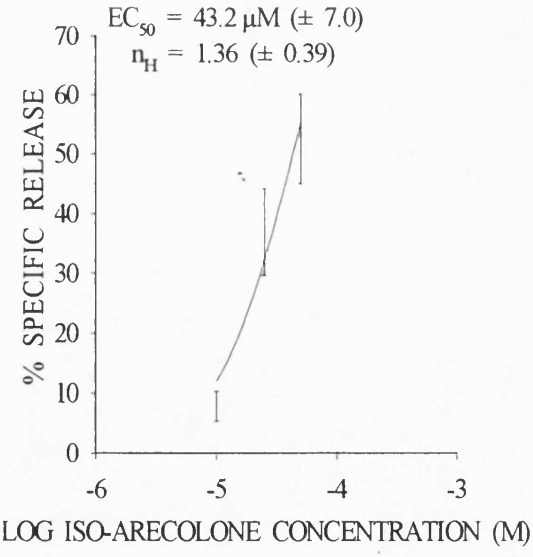


FIGURE 4.10. CURVE FIT TO THE HILL EQUATION FOR ISO-ARECOLONE. The data from table **4.6.** were converted to percent specific release, in each experiment 75 μM iso-arecolone evoked maximal [^{3}H]ACh release, which was taken to be 100%, and then fitted to the non-linear Hill equation to enable an estimate of the concentration at which 50% maximal response was observed.

4.3.4.4. ABT-418

ABT-418 (3-methyl-5-(1-methyl-2-pyrrolidinyl)isoxazole, a novel analogue of (-)-nicotine in which the pyridine ring was replaced with an isoxazole bioisostere (see appendix for structure), is a potent agonist at the α4β2 subtype of neuronal nAChR (Arneric *et al.*, 1994), and became available towards the end of the present study as a gift from Abbott Laboratories (Abbott Park, Illinois, USA). The ability of ABT-418 to evoke [^{3}H]ACh from the hippocampal synaptosome preparation was tested over the range 0.1 to 100 μM, i.e. over a wider range than nicotine or cytisine to allow for any possible reduction in potency. The data obtained, are summarised in table **4.7.** In common with the other agonist dose-response experiments, in each ABT-418 assay, nicotine-evoked [^{3}H]ACh release was used as an internal standard. For each of the other agonists tested, the nicotine standard (either 1 μM or 5 μM), evoked a release of [^{3}H]ACh which was not significantly different from the release obtained in the course of determining the nicotine dose-response curve (section **4.3.2.1.**). If the nicotine-evoked release did differ by more than one standard deviation the whole experiment was disregarded. However it became apparent during the course of determining the ABT-418 dose-response curve that the 5 μM nicotine standard evoked *consistently* low [^{3}H]ACh release (see table **4.7.**). Standard 5 μM nicotine-evoked [^{3}H]ACh release was taken as 904 fmol/mg protein (table **4.1.**), compared to 511 fmol/mg protein obtained in the present series of experiments. It was decided to normalise the ABT-418 release data to enable comparisons with the other agonist dose-response curves. The legitimacy of such an adjustment is discussed in section **4.4.2.**

[ABT-418] (μM)	fmol ACh RELEASED /mg PROTEIN
0.1	170 ± 31.5
1.0	309 ± 44.7
5.0	553 ± 87.8
10	760 ± 28.0
100	389 ± 97
5 μM Nicotine	511 ± 78

TABLE 4.7: THE CONCENTRATION-DEPENDENCE OF ABT-418-EVOKED [^{3}H]ACh RELEASE. Values are fmol ACh released/mg protein (mean ± SEM), corrected for a [^{3}H]Ch uptake of 80 pmol/mg protein/30 min. (*N*=3-4).

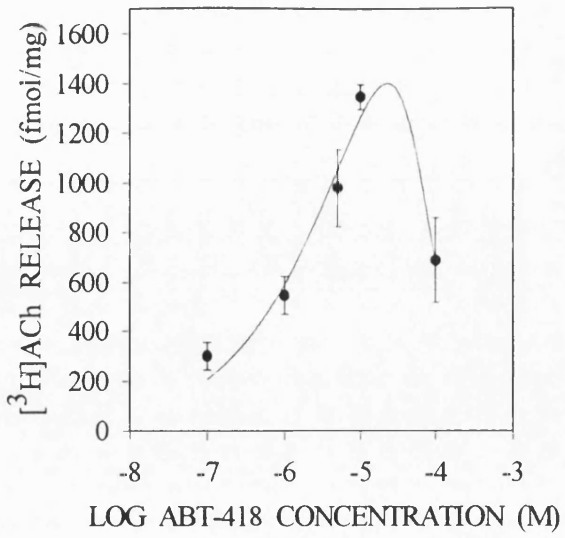


FIGURE 4.11. ABT-418 DOSE-RESPONSE CURVE. Synaptosomes were loaded with [^{3}H]Ch and superfused. After a 45 min washout period, the synaptosomes were stimulated with a 20 s pulse of ABT-418 in Kreb's bicarbonate buffer. Basal release was subtracted to give evoked release which was converted to fmol [^{3}H]ACh and plotted against ABT-418 concentration. Values are mean ± SEM, n = 3-4.

The adjusted data were plotted as figure **4.11.** which shows that ABT-418 in common with the other agonists tested, caused a dose-dependent increase in [^{3}H]ACh release up to the concentration of 10 μM, above which there was a marked attenuation of evoked transmitter release, consistent with ABT-418-induced nAChR desensitisation..

The release data were fitted to the non-linear Hill equation, and the EC_{50} value for ABT-418-evoked transmitter release was calculated as 2.60 ± 0.12 μM

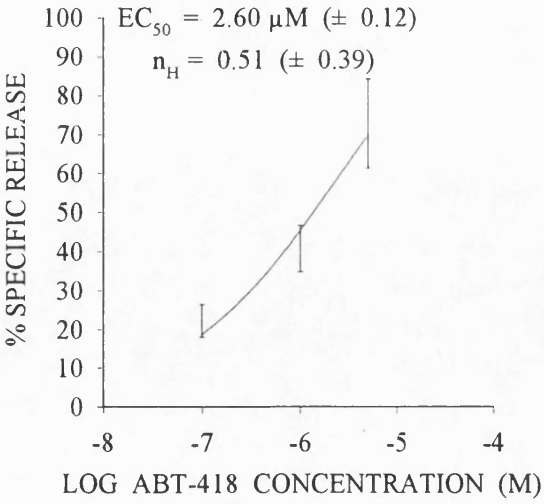


FIGURE 4.12. CURVE FIT TO THE HILL EQUATION FOR ABT-418. The data from table **4.7.** were converted to percent specific release, taking that release recorded at 10 μM ABT-418 to be 100%, and then fitted to the non-linear Hill equation to enable an estimate of the concentration at which 50% maximal response was observed.

The calculated EC_{50} value for ABT-418-evoked [^{3}H]ACh release was 2.60 ± 0.12 approximately two fold less potent than either nicotine- or cytisine at evoking [^{3}H]ACh release. Using the paradigm of nAChR-mediated $^{86}Rb^+$ efflux from mouse thalamic synaptosomes, Marks *et al.*, (1994) have reported an EC_{50} value of 6 μM for ABT-418-evoked efflux, which compares well to the value calculated in the present study.

4.3.4.5. Acetylcholine

An agonist profile of the presynaptic hippocampal nAChR would not be complete without an examination of the responses to acetylcholine (ACh) - the endogenous ligand for the both nAChRs and mAChRs.

The inhibitory role of mAChR autoreceptors on superfused synaptosomes has been characterised: in a series of elegant experiments, Marchi *et al.*, (1981) showed that the presence of exogenous ACh in the superfusion medium produced dose-dependent inhibition of the depolarisation-evoked [^{3}H]ACh release. The EC_{50} of this response was 10 μM ACh. Importantly it was shown that the inhibitory activity of extracellular ACh was inhibited by 0.1 μM atropine (ATR), which itself had no significant effect on the KCl-evoked release of ACh. Thus, to investigate the role of the nAChR in isolation, it is possible to exclude any possible role of mAChR by the addition of ATR. Since the mAChR are inhibitory, one would not expect to observe a change in basal release on application of exogenous ACh via the mAChR. The work of Marchi *et al.*, demonstrated inhibition only of KCl-evoked depolarisation of the synaptosomes.

Therefore, ACh was applied as an agonist in the standard superfusion protocol whereby the loaded synaptosomes were stimulated by various concentrations of ACh forty-five minutes after the start of the superfusion. The concentration of ACh was varied over the range 1 to 500μM, with the results summarised in table **4.8.** and represented graphically in figure **4.13.**

	fmol [^{3}H]ACh released/mg protein				
[ACh] (μM)	1.0	10	50	100	500
S1	88.5	552	835	1357	1615
SEM	46.0	139	311	572	262
n	3	4	3	4	3

TABLE 4.8: ACETYLCHOLINE-EVOKED [^{3}H]ACh RELEASE IN THE ABSENCE OF ATR OR PHY. Values are fmol ACh released/mg protein (mean ± SEM), corrected for a [^{3}H]Ch uptake of 80 pmol/mg protein/30 min. (n=3-4).

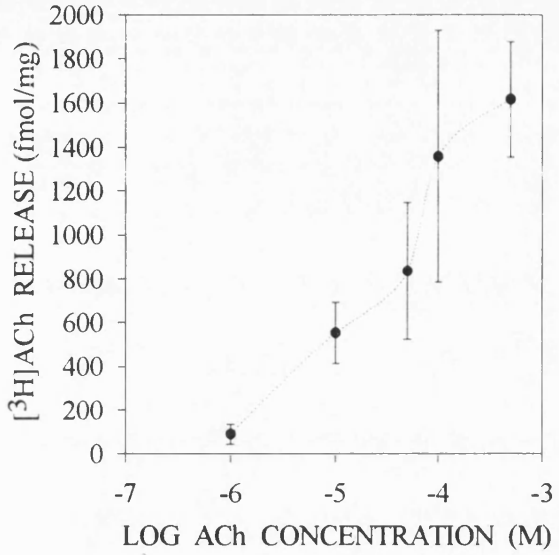


FIGURE 4.13. ACETYLCHOLINE DOSE-RESPONSE CURVE. Synaptosomes were loaded with [^{3}H]Ch and superfused. After a 45 min washout period, the synaptosomes were stimulated with a 20 s pulse of ACh in Kreb's bicarbonate buffer. Basal release was subtracted from agonist-induced peaks to give evoked release which was converted to fmol [^{3}H]ACh at each ACh concentration. Values are mean ± SEM, with n = 3-4.

The resultant dose response curve bears little resemblance to those curves obtained for the other agonists in this study. Maximal [^{3}H]ACh release was evoked at ACh concentrations of between 100 and 500 μM with no obvious desensitisation at higher agonist concentrations and the relatively wide window of activation - the synaptosomes respond to the agonist over almost three orders of magnitude - were clearly unusual.

Despite the stringent washing procedures involved in the isolation of the synaptosomes by the Percoll method, membrane-bound cholinesterases remain in the preparation: cholinesterases which would be sufficient to hydrolyse an unknown proportion of the exogenous ACh pulse. As a result the concentration of ACh actually reaching the nAChRs was likely to be significantly lower than that concentration applied. Hence future perfusions involving ACh as an agonist were carried out in the presence of 0.5 μM ATR

and 1.0 μM PHY. Before further experiments utilising ACh could be determined, it was useful to examine the effects of PHY and ATR on basal release.

Basal release expressed in fmol/mg, was compared at fraction 30 in a large number of experiments in the presence and absence of PHY and ATR. The results are summarised below:

	F30 (fmol/mg) mean ± SEM	
Routine Superfusion buffer:	381 ± 17	(*n*=18)
Buffer + 1 μM PHY + 0.5 μM ATR	389 ± 19	(*n*=35)

Clearly, neither the muscarinic antagonist nor the cholinesterase inhibitor significantly altered the basal release of [^{3}H]ACh, which agrees with the finding of Marchi *et al.*, (1981). However the possibility remains that either atropine of PHY could alter agonist-evoked release of [^{3}H]ACh. To address this possibility, control S1 response to 1 μM nicotine was examined in the presence and absence of ATR and PHY:

	S1 (fmol/mg) mean ± SEM	
1 μM nicotine	865 ± 221	(*n*=5)
1 μM nicotine + 1 μM PHY + 0.5 μM ATR	848 ± 114	(*n*=7)

Again, neither PHY nor ATR significantly alter the agonist-evoked release of [^{3}H]ACh. Confident that the superfusion conditions themselves would not complicate interpretation, the ACh-evoked release experiments were repeated. Anticipating that the PHY would shift the dose response curve, the ACh was tested over the range of 0.5 μM - 50 μM in common with nicotine and cytisine. The initial range of concentrations used was 0.5 μM, 1 μM, 5 μM, 10 μM, and 50 μM, but this was subsequently expanded to include three intermediate concentrations of 2.5 μM, 7.5 μM and 12.5 μM. The results are summarised in table **4.9.**

	[^{3}H]ACh release (fmol/mg protein)							
[ACh] (μM)	0.5	1.0	2.5	5.0	7.5	10	12.5	50
S1	207	1126	1245	423	0	1210	958	804
SEM	47	190	337	61	0	311	151	296
n	4	9	3	8	3	5	3	4

TABLE 4.9: ACh-EVOKED [^{3}H]ACh RELEASE IN THE PRESENCE OF BOTH ATR AND PHY. Values are fmol ACh released/mg protein (mean ± SEM), corrected for a [^{3}H]Ch uptake of 80 pmol/mg protein/30 min..

Judging by the greatly enhanced potency of ACh in the presence of the cholinesterase inhibitor, PHY effectively blocked the action of the endogenous AChE, with the result that full agonist responses (operationally defined as being above 1200 fmol/mg, in common with all the other agonists studied) were observed in the concentration range of 1 μM-50 μM

When these results are plotted, it becomes obvious that the dose response curve does not follow the pattern seen for the other agonists. Rather than an a single inverted 'U' shape indicative of desensitisation at higher agonist concentrations, the dose response curve for ACh-evoked release shows two distinct peaks of release: at 2.5 μM and at 10 μM with a clear and reproducible drop in efficacy at both 5 μM and 7.5 μM.

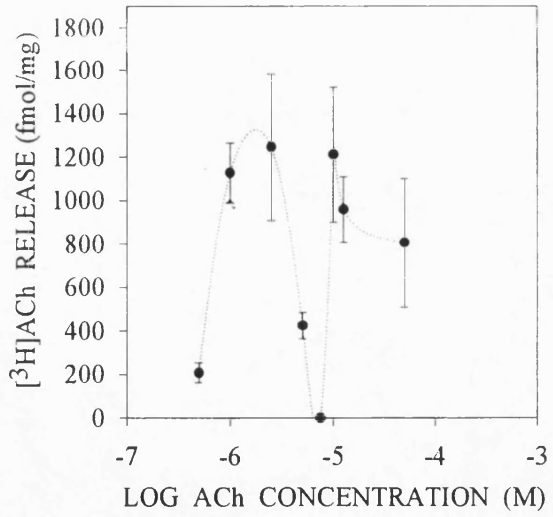


FIGURE 4.14. ACETYLCHOLINE DOSE-RESPONSE CURVE. Synaptosomes were loaded with [^{3}H]Ch and superfused in the presence of 1 μM PHY and 0.5 μM ATR. After a 45 min washout period, the synaptosomes were stimulated with a 20 s pulse of ACh in Kreb's bicarbonate buffer. Basal release was subtracted from agonist-induced peaks to give evoked release which was converted to fmol [^{3}H]ACh at each ACh concentration. Values are mean ± SEM, n = 3-9.

Although such a complex curve precluded the possibility of curve fitting the data to the Hill equation and estimating the EC_{50}, it was possible to estimate very crude values in an analogous manner to that employed for the ANTX dose-response curve. Half-maximal release of [^{3}H]ACh was evoked by ACh concentrations of approximately 0.5-1.0 μM, and 7.5-10 μM.

To ensure that both peaks of release were mediated via nAChR, the nicotinic antagonist DHβE was added to the perfusion buffer at a concentration of 1 μM. As previously described (section **4.3.2.2.**), DHβE has a broad nicotinic specificity and could be expected to block the responses mediated via all nAChR present in the synaptosomal membrane, irrespective of subtype.

Two concentrations of ACh were tested, 1 μM and 10 μM representing both peaks of release. As a internal control, 1 μM nicotine was also tested in each experiment. The results of 3 independent experiments are shown in figure **4.15.**

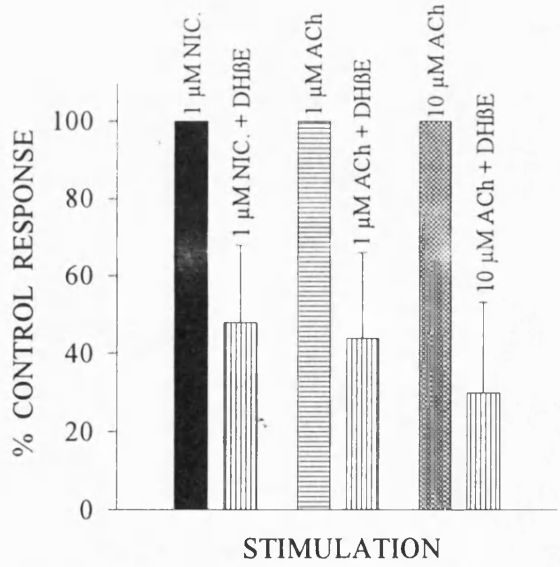


FIGURE 4.15. DHβE BLOCKS BOTH PEAKS OF THE ACh DOSE RESPONSE CURVE. Synaptosomes were loaded with [^{3}H]Ch and superfused. After a 45 min washout period, the synaptosomes were stimulated with a 20 s pulse of 1 μM nicotine, 1 μM ACh or 10 μM ACh in Kreb's bicarbonate buffer in the presence and absence of 1 μM DHβE. Values are mean ± SEM, n = 3. Evoked release for each drug concentration in the absence of DHβE was taken as the 100% control response.

The results indicate that DHβE antagonised the [^{3}H]ACh release evoked by both test concentrations of ACh in addition to the nicotine control. Thus it would appear that the release of [^{3}H]ACh over the concentration entire range of the double-peaked dose response curve was mediated via nAChRs.

4.4. DISCUSSION

4.4.1. CALCIUM DEPENDENCE OF AGONIST-EVOKED [^{3}H]ACh RELEASE

Neuronal nAChR have a substantial permeability to Ca^{2+}, and currents through these neuronal nAChR, (but not muscle nAChR), are modulated by external Ca^{2+}. In contrast to the muscle receptor where external Ca^{2+} decreases the current carried by monovalent cations, in neuronal nAChR ACh-induced currents increase as the extracellular Ca^{2+} concentration increases (Vernino *et al.*, 1992). Application of nicotinic agonists such as nicotine and ACh to CNS neurons (medial habenular neurons) have been shown to cause an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) up to the micromolar range. An increase that was reversibly abolished when Ca^{2+} was removed from the perfusion medium (Mulle *et al.*, 1992). This modulation by external Ca^{2+} is observed even when whole-cell patch-clamped medial hebenula neurons were internally dialysed with simple electrolyte solutions containing 10 mM EGTA or BAPTA which strongly chelate Ca^{2+} (Mulle *et al.*, 1992), therefore external not internal Ca^{2+} accounts for the modulation of the nicotinic agonist-induced response.

In section **4.3.1.** calcium dependence of nicotine-evoked [^{3}H]ACh release was determined as 87.5%. Similar figures for calcium dependency are reported in the literature: Rowell & Winkler (1984) reported that nicotine stimulated [^{3}H]ACh release from mouse cerebral cortical synaptosomes was 58% calcium dependent, a rather low figure perhaps resulting from the use of only 100 μM EDTA to chelate calcium in the superfusion buffer; Rapier *et al.*, (1988) reported a 60% calcium dependency of nicotine-evoked dopamine release from striatal synaptosomes, without the use of chelating agents in the superfusion buffer.

4.4.2. AGONIST-EVOKED [^{3}H]ACh RELEASE

The shape of the agonist dose-response curves was consistent with marked desensitisation of nAChRs at higher agonist concentrations (Marley, 1988) and contrasts with the dose-response curves obtained for example, when studying the ability of nicotine to evoke [^{3}H]dopamine release from striatal synaptosomes (Rapier *et al.*, 1988: Grady *et al.*, 1992). This may reflect differences between nicotinic auto- and hetero-receptors. A bell-shaped dose response curve has been reported by Araujo *et al.*, (1988) who studied

endogenous ACh release from hippocampal and cortical slices in response to nicotine, and found maximal release in the 1 μM to 10 μM range.

The EC_{50} values for nicotine and cytisine compare favourably to values reported in the literature for other presynaptic nicotinic nAChR. For example, for release of [^{3}H]dopamine from rat striatal synaptosomes, Rapier *et al.* (1988) reported an EC_{50} value of 3.8 μM for nicotine-evoked release; whilst in 1992, Grady *et al.* reported EC_{50} values for nicotine- and cytisine-evoked [^{3}H]dopamine from mouse striatal synaptosomes of 0.48 μM and 0.483 μM respectively.

ANTX was approximately seven times more potent than nicotine or cytisine when comparing the EC_{50} values, and twenty times more potent when comparing the concentrations that elicited peak responses. This discrepancy reflects the particularly steep dose-response curve for ANTX compared to the other two agonists. As an agonist at the presynaptic nAChR, ANTX is approximately an order of magnitude more potent than nicotine and cytisine, which is consistent with the literature (Thomas *et al.*, 1993).

Iso-arecolone was approximately forty times less potent than nicotine or cytisine when comparing EC_{50} values and approximately eight times less potent, when comparing the dose required to give a maximal response: a discrepancy again caused by the relative steepness of the dose response curve for iso-arecolone.

Scaling up the ABT-418-evoked [^{3}H]ACh release data according to the magnitude of the internal nicotine standard has a precedent in the literature. Using $^{86}Rb^+$ flux from mouse striatal synaptosomes as an experimental paradigm, Grady *et al.*, (1992; 1994) reported that agonist- and KCl-evoked efflux can vary 300% over many months, necessitating using an internal standard in each experiment, and adjusting the data according to this internal standard if data collected over a considerable time period are to be compared.. The variation in agonist-evoked release was not a problem for most agonists since responses to the internal nicotine standard varied very little. A possible explanation is that the bulk of the agonist dose-response curves (excluding ABT-418) were determined in one six month period, whereas the experiments utilising ABT-418 were carried out over a year later.

The calculated EC_{50} value for ABT-418-evoked [^{3}H]ACh release was 2.60 ± 0.12, approximately two fold less potent than either nicotine or cytisine at evoking [^{3}H]ACh

release. Using the paradigm of nAChR-mediated $^{86}Rb^{+}$ efflux from mouse thalamic synaptosomes, Marks *et al.*, (1994) have reported an EC_{50} value of 6 μM for ABT-418-evoked efflux, which compares well to the value calculated in the present study. Marks *et al.*, (1994) also reported ABT-418-induced desensitisation of the thalamic nAChRs at doses comparable with nicotine-induced nAChR desensitisation in the same preparation, again in agreement with this study. Inverted-'U' shaped dose response curves have also been reported in behavioural studies involving ABT-418-induced retention of inhibitory avoidance training in mice (Decker *et al.*, 1994), suggesting that the relevance of agonist dose-dependency extends beyond *in vitro* biochemical/pharmacological studies.

In summary, one of the obvious things to note about the agonist dose response curves (figures **4.3.**, **4.6.**, **4.8.**, **4.10.**, and **4.11.**) is that all the agonists evoked an approximately equal maximum release of [^{3}H]ACh, in fact none of the maximal responses were statistically different from one another ($p>0.05$; paired 't'-test). So while the potencies of the agonists varied considerably from extremely potent (ANTX) to less potent than nicotine (iso-arecolone), the agonists were equally efficacious, table **4.10.** presents a summary.

	Nicotine	Cytisine	ANTX	Iso-arec	ABT-418	ACh *
Maximal [^{3}H]ACh release (fmol/mg)	1335 ± 92	1456 ± 215	1225 ± 177	1546 ± 371	1345 ± 50	1245 ± 337; 1210 ± 311
[Agonist] producing maximal release	10 μM	10 μM	500 nM	100 μM	10 μM	2.5 μM; 10 μM

TABLE 4.10: COMPARISON OF MAXIMAL EVOKED [^{3}H]ACh RELEASE AND AGONIST CONCENTRATION REQUIRED TO PRODUCE MAXIMAL RELEASE. Data obtained from tables **4.1.**, **4.4.**, **4.5.**, **4.6.**, and **4.9.**, and figure **4.11.** * ACh-evoked release in the presence of ATR and PHY, data from both peaks of [^{3}H]ACh release are quoted.

4.4.3. ANTAGONIST STUDIES

Methyllycaconitine (MLA), the toxic alkaloid from the seeds of *Delphinium spp.* is three orders of magnitude more potent in competition binding assays at the [^{125}I]α-bungarotoxin ([^{125}I]α-Bgt) binding site than at the [^{3}H]nicotine binding site: the K_i reported for inhibition of [^{125}I]α-Bgt to rat brain P2 is of the order of 1 nM (Ward *et al.*, 1990), whilst the K_i for inhibition of [^{3}H]nicotine in the same preparation is 3.7 μM (MacAllan *et*

al., 1988). Thus 1 μM MLA would be expected to block all α7-mediated release without significantly affecting any of the other nAChR subtypes present. 10 μM MLA on the other hand would be expected to significantly inhibit all the nAChR present in the presynaptic membrane.

The inhibition of 10 μM nicotine-evoked [^{3}H]ACh release by the lower concentration of MLA (1 μM) did not reach statistical significance in common with the studies of Drasdo *et al.*, (1992), who reported a non-significant, 16% inhibition of nicotine-evoked [^{3}H]dopamine release from rat striatal synaptosomes. In contrast, 10 μM MLA did significantly inhibit 10 μM nicotine-evoked [^{3}H]ACh release, also in agreement with Drasdo *et al.*, (1992).

The inhibition of the KCl-evoked release was largely independent of MLA concentration and it is possible that the MLA was perhaps able to non-selectively channel block at micromolar concentrations, thus preventing either nicotine or KCl from evoking 100% of the release observed in the control chambers. If approximately 20% of the inhibition observed was independent of the nAChR as indicated by the inhibition of the KCl-evoked release, then it is clear that 1 μM MLA had little inhibitory effect on the nAChR-mediated transmitter release, thus ruling out any major role for the α7 receptor subtype.

4.4.1. POSSIBLE nAChR SUBTYPES IN THE RAT HIPPOCAMPUS

COMPARISON WITH OTHER STUDIES

The subunit composition of the nAChR mediating feedback modulation of ACh release in the hippocampus is unknown, but one candidate is the α4β2 subtype. This defined nAChR, stably expressed in mouse M10 cells (Whiting *et al.*, 1991) was examined using a $^{86}Rb^+$ influx assay (Stephens, 1994; Thomas *et al.*, 1993). The results are similar with the data presented in this chapter.

	EC_{50} (μM)		
nAChR PREPARATION	(-)-NICOTINE	CYTISINE	(+)-ANTX
PRESYNAPTIC nAChRs	0.99 ± 0.23 (4)	1.06 ± 0.15 (3)	0.14 ± 0.04 (3)
α4β2 * nAChR	2.7 ± 0.7 (5)	3.7 ± 1.1 (3)	0.048 ± 0.018 (4)

TABLE 4.11: COMPARISON OF EC_{50} VALUES FOR (-)-NICOTINE, ANTX, AND CYTISINE IN DIFFERENT NEURONAL nAChR PREPARATIONS. Data are mean ± SEM (no. of preparations). * Data from Stephens (1994).

The rank order of potency in both preparations is the same: ANTX > nicotine = cytisine. That the agonist cytisine is as potent as nicotine in the presynaptic hippocampal synaptosome preparation would appear, on first analysis to rule out a role for any β2-containing nAChR subtypes: Luetje & Patrick (1991), found cytisine to be largely devoid of agonist potency at β2-containing nAChR expressed in *Xenopus* oocytes. However, the mouse M10 cells stably express a defined nAChR subtype containing the β2 subunit, and in this preparation the results clearly indicated that cytisine was a full and potent agonist. Cytisine was also examined on α4β2 chick nAChRs using the oocyte expression system by Bertrand (personal communication) and found to be a full agonist with an EC_{50} value of 1.8μM, comparable to that derived for cytisine in both the hippocampal synaptosome and cell expression system. Very recent data suggest that the antagonist DHβE may not have the pan-subunit specificity that was previously envisaged (section **4.3.2.2.**). Luetje *et al.*, (1993) report that in oocytes expressing combinations of α3, β2 and β4 subunits, nAChRs comprising β2 subunits are 50 fold more sensitive to block by DHβE than nAChRs comprising β4 subunits. Indeed, in the reported study, 3 μM DHβE did not produce a statistically significant block in α3β4 subunits. This is interesting because in the present study ACh-evoked release was blocked by 1 μM DHβE, suggesting that the β2 receptor is involved, which is compatible with the *in situ* hybridisation studies outlined below. A note of caution should be added however, because it was the oocyte studies with β2- and β4-containing nAChRs of Luetje & Patrick., (1991), that initially reported a lack of cytisine potency at β2-containing nAChRs.

Compared to nicotine, ABT-418 has reduced potency to interact with the subunit forms of nAChR found in sympathetic ganglia, and it does not compete for α-Bgt sites in brain or at the neuromuscular junction. ABT-418 was reported to be equipotent with nicotine at evoking $^{86}Rb^{+}$ efflux from mouse thalamic synaptosomes (Arneric *et al.*, 1994; Marks *et al.*, 1994), thought to result from the activation of the putative α4β2 nAChRs (Marks *et al.*, 1993), but 10-fold less potent than nicotine at evoking [^{3}H]dopamine release from rat striatal slices, a preparation that has been suggested to involve α3 subunit activation. The observed pharmacology of ABT-418 is also compatible with the presence of α4β2 receptors, in terms of equipotency in evoking [^{3}H]ACh release, compatible with the studies of Arneric *et al.*, (1994).

It is reasonable to assume that the presynaptic nAChR in the hippocampus could contain a β2 subunit, indeed from the rank order of agonist potency, and the comparison of EC_{50} values in the three preparations, the α4β2 subtype would appear to be a good candidate for the presynaptic hippocampal nAChRs.

ACh-EVOKED [^{3}H]ACh DOSE RESPONSE CURVE

A particularly puzzling aspect of the agonist dose response curves, was the shape of the curve evoked by the endogenous agonist ACh. The evidence for two distinct peaks of release is contrary to the studies with the other agonists. One possible explanation is that ACh was activating more than one subpopulation of nAChRs, each with a distinct pharmacology and presumably different affinities for ACh. The major problem with this explanation is that if subpopulations exist, which is perfectly feasible, then why was the differential agonist sensitivity not revealed with any of the other agonists? The answer could lie in the range of agonist concentrations chosen to probe the hippocampal nAChRs. An examination of figures **4.3.** and **4.6.** shows that the dose response curves for nicotine and cytisine are sufficiently broad that they could result from overlapping distinct curves, masking the existence of more than one nAChR subunit combination.

IN SITU EVIDENCE

With respect to the hippocampus, information from *in situ* hybridisation studies can suggest which subunits may be present to contribute to functional nAChR, although these studies are more difficult to interpret in the case of presynaptic receptors. The cholinergic

innervation of the rat hippocampus arises from cell bodies in the medial septum and diagonal band of Broca (Fibiger & Vincent, 1987) (see figure **1.1.**). According to the extensive study of Wada *et al.* (1989), these regions express α4>β2=α2>>α3. Notably, no specific hybridisation of the β4 subunit has been seen in the septum (Dineley-Miller & Patrick, 1992). This would appear to discount the possibility of nAChR containing β4, to explain the observed cytisine efficacy. Perhaps more complex subunit combinations (see section **1.3.2.1.**) including α2, may occur. The α5 subunit has been shown to be associated with α4 and α2 in brain (Conroy *et al.*, 1992) but α5 is not expressed in the septum, as judged by *in situ* hybridisation (Wada *et al.*, 1989).

α7 nAChRs

Although there are few quantitative data available for other subunit combinations, we can confidently discount the α7 subtype: the latter shows much lower sensitivity to agonists (Bertrand *et al.*, 1992; Amar *et al.*, 1993) and the presynaptic autoreceptor is insensitive to α-Bgt (Wonnacott *et al.*, 1989).

SUMMARY

In summary, from the available pharmacological and *in situ* hybridisation data, the candidates for the presynaptic hippocampal nAChR are various combinations of α4, α2 and to a lesser extent α3, in combination with the β2 subunit. The evidence presented in this chapter suggests a strong role for the α4β2 subunit; there are no ligands available to dissect out any contribution by the α2 subunit, though any contribution by α3 could be selectively blocked by neuronal bungarotoxin (nBgt) which is a potent antagonist of α3-containing nAChR whilst only weakly antagonising either α4β2 or α2β2 receptor subtypes (Luetje *et al.*, 1990). Unfortunately, nBgt has not been available to carry out the necessary experiments to delineate any role of α3-containing nAChR subtypes.

4.4.2. AGONIST-EVOKED DESENSITISATION

In a global sense, desensitisation (also known as tachyphylaxis, tolerance, refractoriness, subsensitivity or down-regulation; see Ochoa *et al.*, 1989) refers to the loss of cell or tissue response after an appropriate stimulus is applied repeatedly or for a prolonged period of time. It is well documented the desensitisation of the nAChR can be

triggered by prolonged or repeated exposure to agonists and results in inactivation of the nAChR ion channel (section **1.3.2.4.**).

From the results reported in this chapter, it would appear that each agonist studied is able to induce desensitisation of the nAChRs at sufficiently high concentration, within the duration of the agonist pulse (20 s), yielding inverted-'U' shaped dose-response curves. This would at first seem to be a particularly rapid phenomenon, however Rowell & Hillebrand (1994) reported that nicotine-evoked [^{3}H]dopamine release was subject to desensitisation even at nanomolar concentrations of agonist, and was found to be a first-order process with a half-time of approximately 40 s. Marks *et al.*, (1994) reported ABT-418-induced desensitisation of [^{3}H]dopamine release from striatal synaptosomes, at both non-stimulating (nanomolar) and stimulating (micromolar) concentrations, with a similar rate of onset that increased with increasing agonist concentration. Hence it is conceivable that the lower doses of agonists used in the present study were also able to evoke nAChR desensitisation, but this phenomenon was not apparent because of the relatively short agonist exposure time.

The phenomenon of desensitisation is studied in more detail in the next chapter.

CHAPTER 5 DESENSITISATION STUDIES

5.1. INTRODUCTION

5.1.1. REPETITIVE AGONIST STIMULATION

Using the experimental paradigm of [^{3}H]ACh release from synaptosomes to study the hippocampal nAChRs (chapter 4), it was found that for each of the agonists studied, agonist-evoked transmitter release yielded bell-shaped dose-response curves suggestive of nAChR desensitisation at higher agonist concentrations. The extent of this desensitisation at the various agonist concentrations can be studied by stimulating the synaptosomes twice in succession at each agonist concentration, giving the second agonist stimulus (S2), 30 min after the first (S1). In this way, nAChR desensitisation induced by submaximal concentrations of agonist may be studied. In chapter 4 desensitisation was only apparent at the highest doses of agonist and it was thought that this could be a reflection on the methodology since several groups (e.g. Rowell & Hillebrand, 1994) have reported that nanomolar concentrations of nicotine are able to induce nAChR desensitisation, albeit at a slower rate than at micromolar concentrations.

This procedure of repetitive stimulation is a common pharmacological tool used to study desensitisation. For example, Rapier *et al.*, (1988; 1990) used the technique to study nicotine-evoked [^{3}H]dopamine release from rat striatal synaptosomes The study reported that there was decreased responsiveness with repeated exposure to nicotine, suggesting nAChR desensitisation. Also using [^{3}H]dopamine release as the experimental paradigm, Grady *et al.*, (1994), and Rowell & Hillebrand, (1992; 1994), approached the problem slightly differently. By continuously superfusing synaptosomes in the presence of a wide range of nicotine concentrations, including nanomolar nicotine concentrations that do not themselves elicit transmitter release, both groups showed that nicotine pretreatment attenuates the subsequent ability of a discrete pulse of nicotine to evoke [^{3}H]dopamine release.

In this chapter the effects of repetitive agonist stimulation on the release of [^{3}H]ACh from the rat hippocampal synaptosomes will be examined, by delaying the second stimulation for 30 min, the nAChRs are allowed to recover and the [^{3}H]ACh evoked by the S2 pulse is an index revealing the extent to which this recovery is complete.

5.1.2. SUBSTANCE P

As outlined in section **1.7.**, a number of studies have shown that SP is able to modulate the nAChR-mediated release of neurotransmitters in CNS and PNS tissue preparations, and specifically to influence the rate and extent of nAChR desensitisation. In chapter 4 it was established that at high concentrations, a 20 s pulse of nicotine (S1) is sufficient to produce rapid nAChR desensitisation. There is also the possibility that much lower concentrations are also able to significantly induce desensitisation, which will be investigated in this chapter, at the same time the effects of SP pretreatment on this desensitisation phenomenon will be investigated.

Grady *et al.*, (1992; 1994), and Rowell & Hillebrand, (1992; 1994) used an experimental design in which striatal synaptosomes were superfused with non-activating concentrations of nicotine to induce desensitisation, then stimulated with an activating nicotine concentration. The resultant transmitter release was then compared to that obtained with no nicotine pretreatment. The present study utilised an analogous experimental design, [^{3}H]Ch-loaded hippocampal synaptosomes were superfused in the presence and absence of SP and then challenged with an activating (micromolar) concentration of nicotine (S1). The ability of nicotine to evoke release subsequent to pretreatment with a range of SP concentrations should yield information about the ability of SP to induce nAChR desensitisation in its own right.

In addition to any possible effects on S1 nicotine-evoked [^{3}H]ACh release, the ability of SP to modulate agonist-induced nAChR desensitisation was studied by repeating the repetitive agonist stimulation experiments in the presence of SP. In this way, any changes in the recovery of the nAChR from S1-induced desensitisation would be reflected by changes in the S2/S1 ratio.

5.1.2.1 SP and [^{3}H]nicotine Binding Sites

Further to the superfusion studies mentioned above, the interaction of SP with the hippocampal nAChRs was investigated by conducting [^{3}H]nicotine binding assays. By directly competing SP (10^{-9} - 10^{-5} M) with 20 nM [^{3}H]nicotine, it is possible to assess whether the peptide is acting as a competitive inhibitor at the hippocampal nAChRs. Saturation binding of [^{3}H]nicotine in the presence and absence of SP (10^{-5} M), provides

information as to whether SP affects the K_d or B_{max} of the [^{3}H]nicotine binding in the hippocampus.

5.1.2.2. Nicotine and [^{3}H]SP Binding Sites

An estimation of the density of rat hippocampal SP binding sites was not possible due to the prohibitive cost of tritiated SP ligands - it was not economically viable to carry out more than a pilot saturation study using hippocampal S1 membranes (section **2.1.3.**). Saturation binding with [^{3}H][Sar^9,Met(O$_2$)^{11}]substance P ([^{3}H]SP), a ligand specific for the NK1/SP receptor (see section **2.3.3.**), was estimated in a saturation binding assay with a limited range of [^{3}H]SP concentrations. Reports of SP binding in mammalian brain are scarce, and usually limited to competition binding studies, presumably because the radioligands are so expensive. However functional studies using unlabelled SP have established that NK1 receptors are present in limbic and midbrain dopaminergic areas (Mantyh *et al.*, 1989) where they mediate SP-induced increases in dopamine turnover (Cador *et al.*, 1989). In the locus coeruleus, SP has an excitatory action that is mediated through activation of NK1 receptors (McLean *et al.*, 1991). See also section **1.6.** for further evidence of SP receptors in the CNS.

SP has been reported (Min & Weiland,1993) to interact with two populations of binding sites on the nAChR, a low affinity and a high affinity site (section **1.6.2.2.**). The high-affinity binding site is dependent on the presence of nicotinic agonist (Min *et al.*, 1993), therefore the saturation binding of [^{3}H]SP in the presence and absence of nicotine, was examined, to test whether the equilibrium binding of the tritiated ligand would differ significantly in the presence and absence of nicotine.

5.2. METHODS

5.2.1. REPETITIVE AGONIST STIMULATION

The standard protocol for agonist-evoked release (section **2.2.6.1.**) was adapted as follows: after a 45 min washout period the synaptosomes were stimulated with a test pulse of agonist (nicotine or cytisine; S1); following a 30 min recovery period the synaptosomes were stimulated again with an identical pulse of agonist (S2). The results are expressed in terms of the S2/S1 ratio, corrected in each case for an uptake of [^{3}H]Ch of 80 pmol/mg protein/30 min. In each experiment, a standard pulse of 1 μM nicotine was included as both

S1 and S2, to serve as a control. If the S1 control response differed from the mean 1 μM nicotine response (865 ± 221 fmol/mg protein; section **4.3.2.1.**) by more than one standard deviation, the experiment was disregarded. To control for the effects of [^{3}H]ACh pool depletion, causing an apparent attenuation in responsiveness in the S2 pulse, 20 mM KCl was also used as S1 and S2 test pulses. Since KCl-evoked [^{3}H]ACh release is not mediated by the nAChRs (section **4.3.2.2.**), the extent to which pool depletion causes any apparent decrease in the S2/S1 ratio can be assessed.

5.2.2. SP

Synaptosomes were superfused according to the standard agonist-evoked release protocol (section **2.2.6.1.**), which was adapted as follows: Kreb's buffer included leupeptin (4 μg/ml), chymostatin (2 μg/ml), bacitracin (40 μg/ml), and BSA (200μg/ml). These protease inhibitors and BSA, served to protect SP from proteolytic degradation by endo- and exo-peptidases present in the synaptosomal preparation, and were added after the gassing stage to prevent excessive frothing of the Kreb's buffer. SP was dissolved in the Kreb's buffer. The pH of the buffer was always maintained at 7.4.

To study the effects of SP on the agonist S2/S1 ratio, all chambers were initially superfused with Kreb's buffer containing the protease inhibitors and BSA, in the absence of SP and challenged with a 20 s pulse of 1μM nicotine (S1). The synaptosomes were allowed the usual 30 min recovery period before the S2 pulse, but for the last 6 min, the reservoir buffer was changed and the synaptosomes were superfused in the presence of SP (10^{-6} - 10^{-9} M). This is an adaptation of the protocol used by Khalil *et al.*, (1988), to probe the effect of SP on nicotine induced desensitisation in cultured bovine adrenal chromaffin cells.

The protocols for radioligand binding are detailed in section **2.3.**

5.3. RESULTS

REPETITIVE STIMULATION

5.3.1. REPETITIVE NICOTINE STIMULATION

The effect of repeated stimulation with (-)-nicotine was studied over the concentration range 10^{-7} - $5x10^{-5}$ M and the results are shown in table **5.1.** At a

concentration of 100 nM nicotine, the second agonist stimulus (S2) was equal to 100% of the S1 pulse. As the concentration of nicotine increased, the S2 stimulus declined as a percentage of the S1 response: the S2/S1 ratio declined to approximately 0.25. At every nicotine concentration over 100 nM, the S2 release was significantly smaller ($p<0.01$) than the S1 release. At the highest nicotine concentrations of 10 μM and 50 μM, a subsequent nicotine pulse (S2) was able to evoke only approximately 25% of that release seen with drug-naive synaptosomes (S1).

[Nicotine] μM	S1	S2	S3 20 mM KCl	S2/S1
	[^{3}H]ACh RELEASE (fmol/mg)			
0.1	122 ± 5	122 ± 21	247 ± 22	1.0
0.5	400 ± 71	247 ± 63	200 ± 48	0.62
1.0	820 ± 97	289 ± 34	241 ± 27	0.35
5.0	904 ± 159	288 ± 81	308 ± 41	0.32
10.0	1335 ± 92	277 ± 49	234 ± 22	0.21
50.0	688 ± 191	183 ± 56	272 ± 18	0.27

TABLE 5.1: RELEASE OF [^{3}H]ACh EVOKED BY SUCCESSIVE STIMULATIONS WITH NICOTINE. Values are mean ± SEM, n=3-4

At 50 μM nicotine, the S1 release in absolute terms (688 ± 191 fmol/mg protein), was intermediate between that release observed in response to 0.5 μM nicotine (400 ± 71 fmol/mg protein) and 1.0 μM nicotine (820 ± 97 fmol/mg protein). If pool depletion was completely responsible for the decrease in S2/S1 ratio with increasing agonist concentration, then the S2/S1 ratio for 50 μM nicotine (0.27) should also be intermediate between the S2/S1 ratio for 0.5 μM nicotine (0.62), and that ratio for repetitive stimulation by 1.0 μM nicotine (0.35). This is clearly not the case, and although pool depletion cannot be ruled out as a contributing factor in the decreased S2/S1 ratio, especially at maximal [^{3}H]ACh release (the ratio at 10 μM nicotine: 0.21, exceeds that at 50 μM: 0.27), it would seem likely that agonist-induced receptor desensitisation is a major cause of the attenuation of transmitter release upon repetitive agonist stimulation.

Supporting evidence comes from experiments using 20 mM KCl as the S1 and S2 stimuli. The S2/S1 ratio for 20 mM KCl-evoked release is 1.09 (data from nine

independent assays), arguing against pool depletion playing a major role in the observed attenuation of agonist-evoked release.

At each concentration of nicotine, the synaptosomes were challenged for a third time (S3) with 20 mM KCl. An examination of the results of the S3 stimulus is enlightening. The mean KCl-evoked release was 253 ± 16 fmol/mg protein. Table **5.1.** breaks down the S3 stimulus results according to the concentration of nicotine used in the S1 and S2 challenges; however none of the individual S3 values were statistically different ($p>0.05$) from the calculated mean, providing perhaps the most convincing evidence pool depletion alone is not sufficient to account for the decrease observed in the S2/S1 ratio, assuming that agonists and KCl evoke [^{3}H]ACh release from the same intra-synaptosomal pool. Rowell & Hillebrand (1994) reported that agonist-induced desensitisation recover relatively slowly compared to the onset, and that the recovery from a 20 min exposure to 30 nM nicotine was 85% after 40 min. It is therefore entirely reasonable that using the much shorter exposure time of 20 s, the recovery from 100 nM nicotine-induced desensitisation was complete in the present study. It also seems feasible that recoveries of less than 30% were noted after 30 min using the much higher nicotine concentrations in the range 10 to 50 μM. The release data are represented in figure **5.1.**

The histogram (figure **5.1.**) summarises the effects of repetitive agonist stimulation. The S1 response at increasing nicotine concentrations has been described previously (section **4.3.2.1.**). The S2 responses were also broadly bell-shaped, which is to be expected: as the concentration of S2 nicotine increases, the amount of evoked [^{3}H]ACh release also increased correspondingly, but at nicotine concentrations exceeding 1 μM, the effects of S1-induced nAChR desensitisation were sufficient to cause attenuation of the subsequent S2-evoked response. In addition at the highest nicotine concentrations, the S2 challenge itself was probably able to induce nAChR desensitisation within the 20 s time scale of the pulse. The KCl-evoked [^{3}H]ACh release remained relatively consistent and did not reflect the dose dependency of the S1 and S2 nicotine challenges.

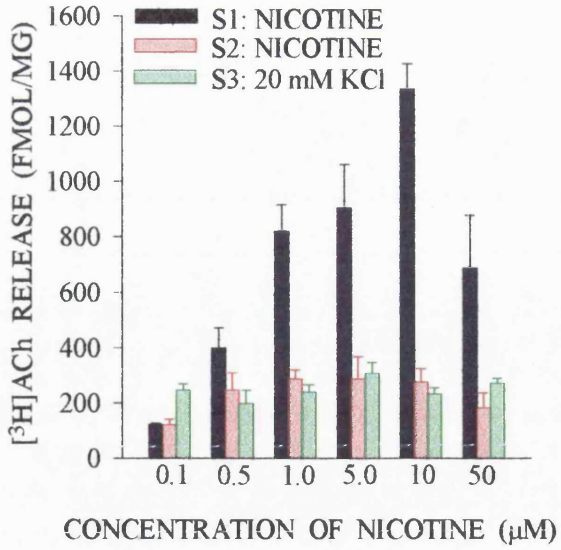


FIGURE 5.1. HISTOGRAM SHOWING THE EFFECTS OF REPETITIVE NICOTINE STIMULATION. Data were taken from table **5.1.**

5.3.2. REPETITIVE CYTISINE STIMULATION

To control for the possibility that the nAChR desensitisation induced by repetitive stimulation, was a phenomenon peculiar to the agonist nicotine, the repetitive agonist stimulation experiments were repeated using cytisine (10^{-7} - 10^{-5} M). The results are summarised in table **5.2.** and mirror the trends observed using nicotine. The S2/S1 ratios generally decrease with increasing agonist concentration, the only anomaly being the particularly low value of 0.21, recorded for a cytisine concentration of 1.0 M, though since the data are from only three independent sets of experiments, this low ratio could be simply experimental variation with three low S2 values at this concentration leading to a falsely low ratio, a possibility that could be examined if further experiments were carried out.

[Cytisine]	S1	S2	S3	S2/S1
μM			20 mM KCl	
	[^{3}H]ACh RELEASE (fmol/mg)			
0.1	160 ± 60	187 ± 20	183 ± 45	1.17
0.5	394 ± 124	309 ± 63	181 ± 38	0.78
1.0	775 ± 127	166 ± 37	239 ± 93	0.21
5.0	993 ± 160	279 ± 70	197 ± 30	0.28
10.0	1456 ± 215	242 ± 58	335 ± 51	0.17

TABLE 5.2: RELEASE OF [^{3}H]ACh EVOKED BY SUCCESSIVE STIMULATIONS WITH CYTISINE. Values are mean ± SEM, n=3.

The mean KCl-evoked S3 release of [^{3}H]ACh was: 237 ± 27 fmol/mg protein. This is not significantly different ($p>0.05$) from the S3 release in the repetitive nicotine stimulation experiments (253 ± 16 fmol/mg protein and none of the individual S3 mean values shown in table **5.2.** are significantly different ($p>0.05$) from this overall mean value. The individual values do not show any trends: it would seem that the release of [^{3}H]ACh evoked by KCl was independent and unaffected by the concentration of the previous nicotine stimuli.

SP STUDIES

5.3.3. SP AND NICOTINE-EVOKED [^{3}H]ACh RELEASE.

To study the effects of SP on S1 release, the synaptosomes were superfused with SP (10^{-6} - 10^{-9} M) dissolved in the Kreb's buffer throughout the duration of the experiment. Nicotine (1 μM) was used as the S1 test pulse in each case. One chamber was always superfused using Kreb's buffer containing the protease inhibitors but no SP, to control for any effects that the protease inhibitors themselves might have on basal and agonist-evoked [^{3}H]ACh release. The results for control S1 (1 μM nicotine), release of [^{3}H]ACh, versus SP-pretreatment are summarised in table **5.3.** At no concentration does SP pretreatment significantly change the nAChR-mediated release of [^{3}H]ACh from hippocampal synaptosomes compared to the control release in the absence of SP.

CONDITION	1 μM NICOTINE-EVOKED ACh RELEASE (fmol/mg)	(n)
Control	654 ± 25	3
10^{-9} M SP	576 ± 81	2
10^{-8} M SP	741 ± 76	2
10^{-7} M SP	647 ± 52	2
10^{-6} M SP	665 ± 43	2

TABLE 5.3: EFFECT OF SP PRETREATMENT ON NICOTINE-EVOKED [^{3}H]ACh RELEASE. Values are the mean ± SEM, or mean ± range, where n = 2.

In section **5.3.1.** the release evoked by 1 μM nicotine was 820 ± 97 fmol/mg, this is significantly larger ($p<0.05$) than the control [^{3}H]ACh release in table **5.3.** of 654 ± 24.9 fmol/mg. The difference between the two protocols was the presence of the protease inhibitors leupeptin, bacitracin, and chymostatin as well as BSA, in the SP experiments.

A possible interpretation is that although SP has no appreciable effect at any concentration on the S1-evoked release, the protease inhibitors and/or the BSA, inhibit nicotine-evoked [^{3}H]ACh release by approximately 20%. The effects of the protease inhibitors on the basal release profile was examined. In a single experiment, six chambers were superfused in the presence, and six chambers in the absence of the protease inhibitors and BSA. At fraction 13, immediately prior to the S1 challenge, the basal release was:

Kreb's buffer: 9947 ± 147 cpm

Kreb's + Inhibs./BSA: 9817 ± 121 cpm

Clearly there is no difference between the basal [^{3}H]ACh in either condition. Furthermore, in section **3.3.3.** the typical basal release at fraction 13 was calculated to be 9271 ± 832 cpm (n=25), and so the release in the presence of the inhibitors was completely typical.

5.3.4. SP AND THE NICOTINE-EVOKED S2/S1 RATIO

All chambers were challenged with an S1 pulse of 1 μM nicotine. The mean evoked [^{3}H]ACh release was 629 ± 25 fmol/mg. The S2 challenge (1 μM nicotine) gave a mean S2 response of 255 ± 16 fmol/mg (n=4) and this was taken to be 100%. A reduction of approximately 20% in the S1 value with only a small, non-significant, reduction in S2 means that the S2/S1 ratio was 0.41, with the apparent increase reflecting the reduced S1 response. The S2 responses following SP pretreatment (10^{-9} - 10^{-6} M; 6 min prior to S2) or

Kreb's buffer were calculated as a percentage of the control response. The results are summarised and the S2/S1 values calculated in table **5.4.**

CONDITION	S2 (% CONTROL)	S2/S1
Control	100%	0.41
10^{-9} M SP	47.1% *	0.19 *
10^{-8} M SP	53.7% *	0.22 *
10^{-7} M SP	60.0% *	0.24 *
10^{-6} M SP	87.8%	0.36

TABLE 5.4: EFFECT OF SP ON THE AGONIST-EVOKED S2/S1 RATIO. Values are the mean for *n*=3-4, SEM were <10% of the mean in each case, * = significantly different from control at $p<0.05$.

At a concentration of 1 μM, SP had an small, non-significant inhibitory effect on this recovery from desensitisation, an effect which became more pronounced as the concentration of SP decreased from 1 μM to 1 nM. This is discussed in the section **5.4.2.**

5.3.5. [^{3}H]NICOTINE BINDING AND SP

5.3.5.1. Competition Binding

The effects of SP on the equilibrium binding of 20 nM [^{3}H]nicotine to rat hippocampal S1 membranes are summarised in table **5.5.** At no concentration of SP is the[^{3}H]nicotine binding significantly different ($p>0.05$) from the control.

CONDITION	% CONTROL BINDING	(n)
Control	100%	4
10^{-9} M SP	103%	4
10^{-8} M SP	105%	4
10^{-7} M SP	119%	3
10^{-6} M SP	99.2%	3
10^{-5} M SP	103%	4

TABLE 5.5: EFFECT OF SP ON THE EQUILIBRIUM BINDING OF 20 nM [^{3}H]NICOTINE. Values shown are the mean, SEM <10% at each concentration. Control (binding in absence of SP) defined independently in each individual assay. 100% control binding = 20.0 ± 5.8 fmol/mg protein. Protease inhibitors were included in the assay buffer to stabilise SP (section **2.3.1.2.**).

It appears unlikely that SP is an inhibitor of [^{3}H]nicotine binding to hippocampal membranes, though it is possible that no apparent inhibition was observed because the range of concentrations of SP was too narrow. Min & Weiland (1993) described no SP inhibition of ^{125}I-α-Bgt binding to *Torpedo* membranes, until the SP concentration exceeded 100 μM. However such high concentrations of peptide are probably unphysiological and not relevant to the superfusion studies described above, where the highest concentration of SP used was 10 μM. SP concentrations in rat brain are reported to be approximately 100 pmol/gram brain tissue (McGeer, 1987) compared with approximately 25 nmol/gram, for ACh. Thus it is likely that endogenous concentrations of SP would not exceed high nanomolar/low micromolar.

5.3.5.2. Saturation Binding

The approximate EC_{50} value determined in functional studies of SP inhibition of nAChR activation (Boyd & Leeman, 1987; Simasko *et al.*, 1987; Min & Weiland, 1992), is 10 μM SP. This concentration of SP was used by Min & Weiland (1993), who determined that at 10 μM, SP increased the affinity of [^{3}H]ACh binding to *Torpedo* membranes approximately threefold, from 250 ± 60 nM to 85 ± 2 nM. In six independent experiments carried out in triplicate, the saturation binding of [^{3}H]nicotine was examined in the presence and absence of 10 μM SP. The results are shown in table **5.6.**, figure **5.2.** is a representative saturation isotherm.

CONDITION	K_d (nM)	B_{max} (fmol/mg protein)
CONTROL	4.4 ± 1.5	29.9 ± 5.4
+ 10 μM SP	5.2 ± 1.2	30.1 ± 5.9

TABLE 5.6: EFFECT OF SP ON SATURATION BINDING OF [^{3}H]NICOTINE TO HIPPOCAMPAL S1 MEMBRANES. Values are the mean ± SEM for n=6. K_d and B_{max} values determined from Scatchard analysis.

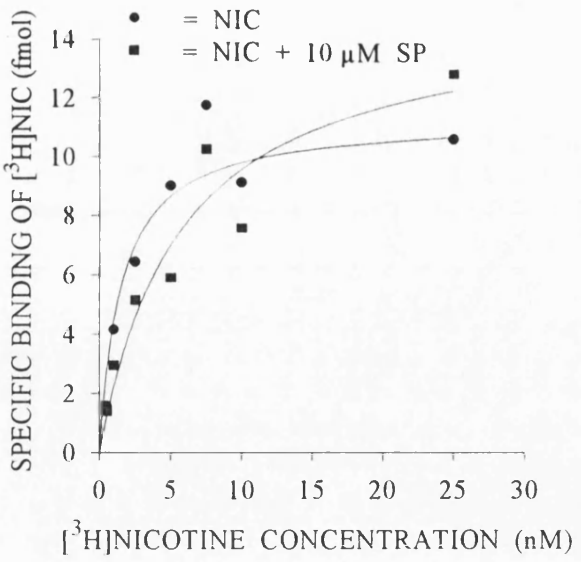


FIGURE 5.2. A REPRESENTATIVE ISOTHERM SATURATION BINDING OF [^{3}H]NICOTINE TO HIPPOCAMPAL S1 MEMBRANES. Membranes were incubated with increasing concentrations of [^{3}H]nicotine (0.5 - 25 nM) as described in section **2.2.1.**

At a concentration of 10 µM, SP did not significantly affect either the observed maximum number of binding sites, or the apparent affinity of the agonist for these sites.

5.3.6. [^{3}H]SP BINDING AND NICOTINE

5.3.6.1. Saturation binding

A single [^{3}H]SP saturation binding experiment was performed in triplicate in order to estimate the approximate B_{max} and K_d of NK1/SP binding sites in the hippocampal S1 membrane preparation.

The experiment yielded the following saturation curve (figure **5.3.**) with an approximate K_d of 20 nM and B_{max} of 80-100 fmoles/mg protein. This crude estimation agrees rather well with other reported studies such as Utkin *et al.*, (1989), who reported a binding density of 60 ± 10 fmoles/mg protein, and a K_d of 9.0 ± 6.0 nM using [^{125}I]BH-SP as a ligand to probe NK1/SP sites in rat brain membranes. According to Quirion & Dam (1993), [^{3}H]SP apparently binds to a single class of high affinity (K_d = 1.4 ± 0.5 nM), low capacity (B_{max} = 160 ± 3.0 fmol/mg protein) sites in rat brain homogenates. The same authors report that at concentrations approximating K_d values, specific binding reached rapid equilibrium (< 30 min) at 25°C and represented between 70 and 75% of total binding. The ligand [^{3}H]SP specifically and selectively labels the NK1/SP receptor subtype since only NK1 competitors such as substance P and unlabelled [Sar^9,Met(O$_2$)^{11}]substance P behave as potent competitors (K_i in low nanomolar range) in this assay whereas selective

NK2 ([Nle^{10}]NKA$_{4-10}$) and NK3 (senktide) analogues are virtually inactive (Dam *et al.*, 1990). As a first approximation, the single saturation experiment reported in this study yields K_d and B_{max} values in the right range, it is also possible that the density found in rat brain homogenates as a whole reported by Quirion & Dam would vary from one brain region to another.

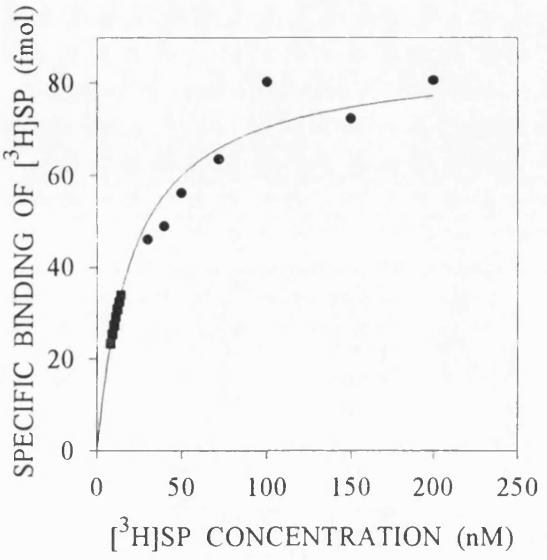


FIGURE 5.3. SATURATION ISOTHERM FOR THE BINDING OF [^{3}H] SP TO HIPPOCAMPAL S1 MEMBRANES. Membranes were incubated with increasing concentrations of [^{3}H]SP (0.01 - 200 nM) as detailed in section **2.3.3.1.**

The B_{max} for [^{3}H]SP binding was approximately three times greater than the B_{max} for [^{3}H]nicotine binding sites. Even allowing for high affinity SP binding to the nAChRs (1 SP site/nAChR; Min *et al.*, 1994), the density of SP/NK1 receptors greatly exceeds the density of the nAChRs. According to Min *et al.*, (1994), SP binding to the nAChRs is increased 300 - 400% if the binding assay is performed in the presence of nicotinic agonist. Hence a [^{3}H]SP competition assay versus nicotine was conducted.

5.3.6.2. Competition Binding

Binding of [^{3}H]SP at its approximate K_d value (20 nM) to hippocampal S1 membranes was carried out in the presence and absence of increasing concentrations of nicotine (10^{-10} - 10^{-6} M). A single competition assay was performed in triplicate. The results are shown in table **5.7.** A 300 - 400% increase in binding would be rather obvious but was absent, and although this was only a single experiment, it seems unlikely that the SP ligand was binding to the hippocampal nAChRs, or if it was, the binding was unchanged in the presence of a variety of concentrations of nicotinic agonist.

CONDITION	% CONTROL BINDING
CONTROL	100%
10^{-10} M NICOTINE	102%
10^{-9} M NICOTINE	92.4%
10^{-8} M NICOTINE	104%
10^{-7} M NICOTINE	108%
10^{-6} M NICOTINE	89.1%

TABLE 5.7: COMPETITION BINDING OF [^{3}H]SP AGAINST NICOTINE. Values are the mean of 3 observations in 1 experiment. 100% binding = 45.5 ± 8.7 fmol/mg (total cpm = 1269, non-specific = 463).

5.4. DISCUSSION

5.4.1. REPETITIVE AGONIST STIMULATION

The [^{3}H]Ch-loaded synaptosomes were subject to two agonist (nicotine or cytisine) challenges at thirty minute intervals, S1 and S2. The same concentration of agonist was used for both stimuli, and it was found that with increasing concentrations of agonist, the ability of a successive agonist challenge, S2, to evoke [^{3}H]ACh release was increasingly attenuated as measured by the ratio S2/S1.

Rapier *et al.*, (1988) concluded that repeated exposure to agonist (nicotine) caused desensitisation of the presynaptic nAChR modulating [^{3}H]dopamine release from striatal synaptosomes. In a more extensive study in 1990, the same authors (Rapier *et al.*, 1990) found that a second stimulation of agonist (nicotine, ACh, and carbamylcholine) at high concentration (100 μM), resulted in an S2/S1 ratio of between 0.38 and 0.50. These results are concordant with the present study given the differences in brain region, transmitter, and

possible differences in the propensity of the two different presynaptic nAChRs (striatal heteroreceptor compared with hippocampal autoreceptor) to desensitise, which may be a property inherent to the particular nAChR subtype. Using mouse striatal synaptosomes, Grady *et al.*, (1994), determined that repetitive stimulation with micromolar concentrations of nicotine caused an attenuation in the release of [^{3}H]dopamine of up to 75%, i.e. the minimum S2/S1 ratio was 0.25, very close to the value reported for nicotine (0.21) and cytisine (0.17) in this study. The further decrease below 0.25 might be either the contribution of pool depletion from the hippocampal synaptosomes, or again differences in species, brain region and technique. Grady *et al.*, (1994) calculated rates of recovery from desensitisation by varying the length of time between the S1 and S2 pulses. Though not directly comparable because the authors used differing concentrations of nicotine for the S1 (3 μM) and S2 (10 μM) pulses, the calculated half time ($t_{½}$) of recovery from micromolar nicotine-induced desensitisation was approximately 12 min. This is very different from the rate of recovery of the hippocampal nAChRs: after a 30 min recovery period, at every nicotine or cytisine concentration over 1 μM, there was still considerably less than 50% recovery (S2/S1 ≤ 0.35), suggesting that the $t_{½}$ for recovery is considerably greater than 12 min, indeed at agonist concentrations exceeding 5 μM, the ability of a subsequent agonist challenge to evoke the release of [^{3}H]ACh, was still maximally attenuated (S2/S1 ≤ 0.25). As well as calculating $t_{½}$ for recovery from micromolar agonist-induced desensitisation, Grady *et al.*, also pretreated their synaptosomes for 20 minutes with 30 nM nicotine, allowed varying lengths of recovery time and then studied the release of [^{3}H]dopamine evoked by a subsequent stimulus of 10μM nicotine. In this way the authors calculated that the $t_{½}$ for nanomolar agonist-induced desensitisation was approximately 6 min. The fact that desensitisation was observed following exposure to nanomolar agonist indicates that in the present study the S2/S1 ratio for 100 nM concentrations of cytisine and nicotine (1.17 and 1.00 respectively) could either indicate no desensitisation or recovery of the nAChRs, within the 30 min interval between S1 and S2 stimulations.

5.4.2. POOL DEPLETION

Depletion of the releasable pool of transmitter has a confounding effect on the interpretation of nAChR desensitisation in this study. Within the synaptosome, ACh is stored in two metabolically different pools (reviewed by Solsona *et al.*, 1991; see also Dolezal *et al.*, 1993), the bound pool is associated with synaptic vesicles and the so-called free pool may represent ACh associated to a specially labile fraction of synaptic vesicles, or it may correspond to the cytoplasmic fraction of ACh. Using vesamicol, a potent inhibitor

of ACh uptake into isolated synaptic vesicles, various reports (e.g. Anderson *et al.*, 1983) have shown inhibition of recently synthesised ACh from *Torpedo* synaptosomes while being ineffective on release of preformed ACh (Michaelson *et al.*, 1986). This observation was taken was taken as support for the vesicular origin of the released transmitter. Disruption of the plasma membranes by homogenisation and/or freezing and thawing does not affect the synaptic vesicles, hence estimations of the bound versus free ACh can be made. The bound ACh compartment, protected from hydrolysis by acetylcholinesterases, comprises 60-70% of total ACh and has a slow turnover (Dolezal *et al.*, 1993). Dolezal *et al.*, (1993) labelled this bound pool using 10 μM [^{14}C]Ch and reported that the ACh synthesised per hour represented 7% of the total ACh content of the synaptosome.

In the present study it is expected that following uptake of [^{3}H]Ch into the synaptosome, the labelled precursor is acetylated to form [^{3}H]ACh by the enzyme ChAT, and transported into the synaptic vesicles.. Following the 30 min incubation, the synaptosomes were washed to remove any unbound [^{3}H]Ch and then superfused for a washout period of 45 min to achieve a stable transmitter baseline before the start of each experiment. Hence, at the start of the superfusion, [^{3}H]ACh is expected to comprise less than 10% of the bound pool of ACh (see above), the pool which is preferentially released upon depolarisation (Solsona *et al.*, 1991; Dolezal *et al.*, 1993). Since there is no free [^{3}H]Ch to continue synthesis of [^{3}H]ACh during the course of the superfusion experiment, the pool of releasable [^{3}H]ACh is finite and can be expected to decline in two ways. Firstly, approximately 1% of the total [^{3}H]ACh content of the synaptosomes is released per 3 min fraction (section **3.3.4.**), constituting basal release, hence in a typical 30 fraction experiment, approximately 30% of the total pool is depleted, by the end. Ten fractions typically separate the first and second agonist stimuli (S1 and S2), hence basal pool depletion would be expected to contribute 10% to any decrease in S2/S1 ratio. Secondly, the labelled pool would be depleted following evoked release: maximal agonist-evoked release never exceeded 3% of the total tissue content however, so the maximum decrease in S2/S1 due solely to evoked pool depletion would be approximately 3%. Altogether, pool depletion would be unlikely to account for more than a 10 - 13% reduction in S2/S1 ratio in the present system. Further reductions would therefore appear to implicate nAChR desensitisation/inactivation.

5.4.3. EFFECTS OF SP ON NICOTINE-EVOKED [^{3}H]ACh RELEASE

Livett & Zhou (1991) summarised the actions of SP, with regard to nicotinic stimulation of catecholamine (CA) release from bovine adrenal chromaffin cells. At SP concentrations > 10^{-7} M, the peptide inhibited CA secretion evoked by non-desensitising concentrations of nicotine in a dose-dependent manner (Khalil *et al.*, 1988), however at high concentrations of nicotine, SP (10^{-9} - 10^{-5} M) protected against nicotinic desensitisation, an effect which outweighed the inhibition of release, and led to facilitated CA secretion (Khalil *et al.*, 1988). Lyford *et al.*, (1990) reported directly conflicting results. Also working with CA release from bovine chromaffin cells, the authors reported that the rate of desensitisation of CA release due to stimulation with a nicotinic agonist appeared to be enhanced in the presence of SP and diminished immediately following its removal. In addition, the removal of SP was reported to lead to a delayed and relatively slow phase of CA secretion. To further cloud a rather muddy issue, as long ago as 1981, Role *et al.*, reported that preincubation with SP did not affect the secretion of CA during a subsequent nicotinic challenge in cells from guinea-pig adrenal medulla.

How does the literature compare with the transmitter release studies in sections **5.3.3.** and **5.3.4.**? The protease inhibitors used to protect the SP and/or the BSA in the Kreb's buffer depressed S1 nicotine-evoked [^{3}H]ACh release from the hippocampal synaptosomes, but no further effect was seen in the presence of any concentration of SP. The peptide does affect the S2/S1 ratio in repetitive agonist stimulation experiments, but in rather a complicated way. At 10^{-9} M, SP may have enhanced the desensitisation of the nAChRs, apparently causing a significant decrease in the control S2/S1 ratio from the control value of 0.41 to 0.19, which would agree with Lyford *et al.*, (1990). At concentrations of 10^{-8} M and 10^{-7} M, SP also significantly enhanced nAChR desensitisation, though to a slightly lesser extent than the 10^{-9} M concentration. At 10^{-6} M, SP did not significantly enhance desensitisation compared to the control situation. In the experimental protocol, the synaptosomes were pretreated with SP for the 6 min immediately prior to the S2 nicotine challenge, after which the synaptosomes were superfused with regular Kreb's buffer. Typically, the peak of agonist-evoked release was collected in the 2 fractions (6 min) following the challenge (see chapter 3). It is feasible that removal of the peptide provokes a 'delayed and relatively slow phase of secretion' as reported by Lyford *et al.*, (1990). The secretion would overlay any apparent enhanced desensitisation of the nAChRs, and as the concentration of SP increased the secretion would slowly offset the decrease

seen in the S2/S1 ratio, which was indeed what was observed. It remains to be seen whether the S2/S1 ratio would continue to increase and perhaps surpass the control value, if the concentration of SP was increased sufficiently, and this is certainly an area which would be amenable to further investigation. There are a number of possible ways that SP could provoke transmitter release, though necessarily through no direct action on the agonist binding site on the hippocampal nAChRs, as discussed below.

5.4.4. RADIOLIGAND BINDING, NICOTINE AND SP

5.4.4.1. [^{3}H]Nicotine Binding

The values for the binding affinity (K_d = 4.4 nM) and binding density (B_{max} = 29.9 fmol/mg protein) of [^{3}H]nicotine are in agreement with the literature (reviewed in Wonnacott, 1987):

K_d	B_{max}	Preparation/Author
2 nM	65 fmol/mg	(hippocampal P2; Yoshida & Imura, 1979)
2.9 nM	7.3 fmol/mg	(rat hippocampus homogenate; Benwell & Balfour, 1985)
5.3 nM	47 fmol/mg	(rat brain P2; Nukina *et al.*, (1985)
9 nM	108 fmol/mg	(rat brain P2; MacAllan *et al.*, 1988)
3.6 nM	57 fmol/mg	(rat brain P2; Martino-Burrows & Kellar, 1987

SP did not significantly affect either the observed maximum number of binding sites, or the apparent affinity of the agonist for these sites at a concentration of 10 μM, but this may not be surprising since Min *et al.*, (1994) reported that photoaffinity labelling of Torpedo membranes with an iodinated SP ligand (^{125}I-[Phe^8(pBz)]SP) in the presence of 100 μM unlabelled SP to block the neurokinin receptors, led to no specific labelling of the α or β subunits of the *Torpedo* nAChRs. Rather, the δ subunit was specifically labelled and to a lesser extent, the γ subunit and the authors speculated that the affinity labelling was consistent with a binding site within the ion channel pore, where the δ and γ subunits are in closest proximity.

However a direct interaction of SP with rat α3, α4 and β2, β4 subunits expressed pairwise in *Xenopus* oocytes has been reported by Stafford *et al.*, (1994). The peptide was found to be a noncompetitive inhibitor of all pairwise combinations of subunits expressed,

though the IC_{50} values for inhibition of ACh-induced current, differed markedly depending on the β subunit. The IC_{50} values for β4-containing receptors was in the range of 2.5 - 3.5 μM, whereas the values for β2 containing receptors was between 65 and 75 μM. The α subunit that was coexpressed had no significant effect on the apparent affinity for SP. Since the inhibition by SP was not voltage dependent it is less likely that in the receptors expressed in the oocytes at least, the SP is physically blocking the channel. If the positively charged peptide entered the channel deeply enough, the inhibition should have been sensitive to the potential across the membrane. However, the possibility cannot be ruled out that SP may block at the extracellular mouth of the channel, outside of the potential field of the membrane.

In both *Torpedo* membrane nAChRs and coexpressed α/β hetero-oligomeric nAChRs expressed in oocytes, SP acts as an inhibitor, and in the present study there was no evidence to suggest an inhibitory action on [^{3}H]nicotine binding to hippocampal membranes.

In summary, SP did not alter the apparent binding density of [^{3}H]nicotine to hippocampal membranes, nor the affinity of the nAChRs for the ligand, therefore despite various reports in the literature using *Torpedo* membrane preparations and in oocyte-expressed rat nAChRs, it would appear that SP did not act as an inhibitor of [^{3}H]nicotine binding in rat hippocampal membranes.

5.4.4.2. [^{3}H]SP Binding

How can SP affect the S2/S1 ratio, if there was no evidence for direct interaction with the agonist binding site of hippocampal nAChRs? The saturation binding experiment with the NK1 ligand [^{3}H]SP indicates the presence of NK1 receptors in the rat hippocampus. SP does not affect nicotine-evoked transmitter release directly via the nAChRs, Valenta *et al.*, (1993) summarised evidence showing that SP did not directly block the nAChR channels of chicken sympathetic ganglia, indeed Simmons *et al.*, (1990) showed that SP was able to modulate nAChR channels in cell-attached patches when the peptide was applied to the *extra*-patch membrane, i.e. under conditions where the nAChRs channels were not exposed to the peptide at all.

Thus SP could modulate nAChRs via the endogenous tachykinin receptors. Activation of these receptors which are G protein coupled, may lead to increased levels of

intracellular cAMP, which in turn may result in changes in the phosphorylation state of the nAChRs, an important determinant in the desensitisation state of the receptors (Huganir *et al.*, 1986). Activation of the tachykinin receptors may also result in the direct modulation of transmitter release via G protein-mediated increases in triphosphospoinositol (IP_3)/diacylglycerol, mobilisation of Ca^{2+}/activation of protein kinase C (PKC), and phosphorylation of synapsin leading to synaptic vesicle exocytosis and transmitter release. A number of lines of evidence implicate phosphoinositide (PI)/PKC as the likely second messenger mediating SP modulation of nAChR desensitisation. First activators of PKC kinase precisely mimicked the effects of SP on nAChR function (Downing & Role, 1987). Second both PI turnover and PKC activation was detected within seconds of SP treatment of neurons (Valenta *et al,*. 1993). Most compelling is the observation that SP modulation of nAChRs was completely blocked by PKC inhibitors (Simmons *et al.*, 1990). It is not yet clear how SP stimulates PKC in these neurons, although recent studies of mast cells demonstrate a variety of cationic amphophilic peptides, including SP that directly activate G_i and G_o in a receptor- independent manner, stimulating PI hydrolysis and, presumably, PKC activation (Mousli *et al.*, 1990). SP may activate PKC and hence modulate hippocampal nAChRs by a similar mechanism.

In summary, SP did not interact with the agonist binding site on the hippocampal nAChRs, changing neither the affinity of the nAChRs for the ligand [^{3}H]nicotine, nor changing the apparent density of hippocampal [^{3}H]nicotine binding sites. SP did appear to modulate the nicotine-evoked release of [^{3}H]ACh in a complex manner consistent with a possible enhancement of agonist-induced desensitisation/inhibition of recovery from desensitisation, counterpointed by an apparent facilitatory action of SP on the hippocampal synaptosomes, causing [^{3}H]ACh release. The presence of hippocampal SP/NK1 receptors, demonstrated by binding experiments with the ligand [^{3}H]SP, provide an indirect pathway by which SP may effect nAChR modulation, involving the stimulation of second messenger systems.

CHAPTER 6 MICRODIALYSIS

6.1 INTRODUCTION

6.1.1. *IN VIVO* METHODS FOR STUDYING NEUROTRANSMITTER RELEASE

6.1.1.1. Cup Perfusion Technique

The cup perfusion method for the study of *in vivo* transmitter release in the brain, was pioneered in the 1950's by MacIntosh & Oborin (1953). A small cylinder is placed on the surface of the exposed brain, usually the cortex. The cup formed by the cylinder and the cortical surface is perfused with a physiological solution. Endogenous compounds diffuse from the underlying tissue into the cup solution . This method has been extended to freely moving animals using chronically implanted cups (Beani *et al.*, 1968), and until recently the cup technique was the most frequently applied method in physiological and pharmacological studies involving the release of ACh in the brain (Pepeu, 1977; Moroni & Pepeu, 1984). The major drawbacks of the cup perfusion technique include the limited applicability to surface areas of the brain and the large areas that are perfused.

6.1.1.2. Push-Pull Perfusion Technique

Since the first description of the technique by Gaddum (1961), several types of push pull cannulae have been developed. Usually two, often concentric, cannulae are implanted in a localised brain area. Fluid is driven by a perfusion pump through the protruded inner tube and removed through the outer tube. At the tip of the cannula the fluid streams through the surrounding tissue, and substances are taken up in the perfusion fluid . Finally, the perfusates are analysed for the compounds of interest. This technique has been applied to measure the release of ACh from various discrete brain areas (Pepeu, 1977; Philippu, 1984).

Push-pull perfusions may also be used for infusing exogenous compounds to local brain areas, and in contrast with the cup technique, structures deep within the brain can be sampled. Tissue damage may occur however, both during the implantation of the cannulae

and during the push-pull perfusions, due to the continuous mechanical contact of the perfusion fluid and the brain tissue (Yaksh & Yamamura, 1974; Nieoullon *et al.*, 1977).

6.1.1.3. In Vivo Microdialysis

Brain dialysis is a recent addition to the technical armamentarium of the neuropharmacologist for the study of the mechanisms of action and preclinical profiles of centrally acting drugs. After a slow beginning, the brain dialysis technique was improved by the introduction of thin dialysis fibres (Zetterstrom *et al.*, 1983). Subsequently, extensive pharmacological investigations validated its application to the study of dopamine release *in vivo* (Imperato & Di Chiara, 1984; 1986). Brain dialysis is now routinely applied to other neurotransmitters, including excitatory amino acids (Lehman *et al.*, 1983), GABA (Kehr & Ungerstedt, 1988), noradrenaline (L'Heureux *et al.*, 1986), 5-HT (Carboni & Di Chiara, 1989), and acetylcholine (Damsma *et al.*, 1987).

The basic principle is the positioning of a membrane that allows free diffusion of water and solutes between the solution of interest (brain interstitial space) and a solution lacking the substances concerned which is constantly renewed and sampled for analysis of the substance(s) of interest.

In brain dialysis, a dialysis fibre (0.3-0.5 mm diameter) is implanted in a selected brain region. Low molecular weight compounds diffuse down their concentration gradients from the brain extracellular fluid into a physiological salt solution that flows through the fibre at a constant rate. The fluid is collected and analysed. This technique allows many of the substances in the extracellular fluid to be monitored, provided that a sensitive method of detection is available. Brain dialysis has taken advantage of the high sensitivity and selectivity of HPLC with electrochemical or fluorimetric detection, which enables rapid analysis of brain dialysates; the dialysis fibre can even be connected directly to the HPLC for on-line determination (Abercrombie *et al.*, 1989).

The major advantages of brain dialysis over other techniques for *in vivo* monitoring of chemical changes in the brain (cup technique, push-pull cannula) stem from the fact that it is a closed system in which there is no direct contact between the superfusion fluid and the tissue; thus local tissue damage is reduced and relatively clean samples are produced for direct chemical analysis. Indeed, although some dialysis probes (e.g. U-shaped probes) can cause damage, detailed histochemical examination of transcerebral probes *in situ* indicates

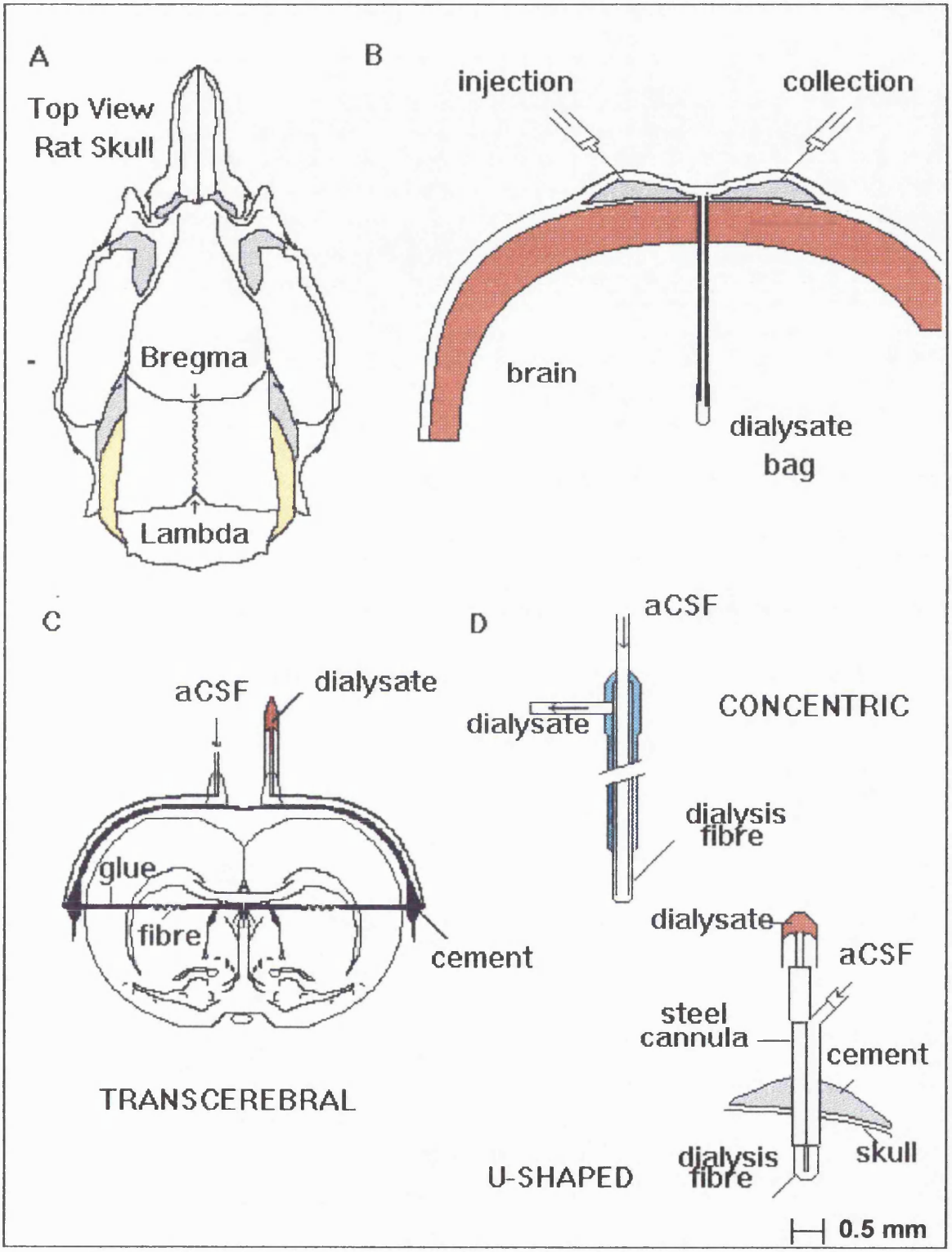
that the degree of tissue damage is minimal (Leone & Di Chiara, 1987). Brain dialysis can be applied to freely moving rats so that behaviour can be correlated with chemical changes in the brain (Imperato *et al.*, 1986).

The first microdialysis model (Bito *et al.*, 1966), consisted of a permeable sack 8-12 mm long and filled with 6% dextran in saline, and had one major drawback - only one sample could be collected per probe. Repeated sampling was developed by Delgado *et al.*, (1971). The same group, a year later, pioneered continuous sampling for assessing neurotransmitter levels in conscious Rhesus monkeys for up to 8 months (Delgado *et al.*, 1972). It was an adaptation of the push-pull cannula technique, which they termed a transdermal dialytrode since it could incorporate a totally subcutaneous stimulating/recording electrode (see figure **6.1.**). The probe was formed from two pieces of Teflon tubing cemented together, with one tip ending 1 mm shorter than the other; both tips were enclosed in a polysulphone bag (3 μl). The other ends of the tubing terminated separately in two rubber reservoirs. The system was continuously superfused and drugs could be injected into one bag and sample collected from the other.

U-shaped and transcerebral probes were developed in the early 1980's, by Zetterstrom & Ungerstedt, who were also one of the first groups to couple the technique to the highly sensitive detection ability of high performance liquid chromatography (HPLC) linked to either electrochemical or fluorometric detection. It was only with the advent of HPLC that the full potential of measuring neurotransmitters from small samples of extracellular fluid could be exploited. Transcerebral and U-shaped probes are not suitable for use in deep brain structures such as the hypothalamus, since their implantation causes too much damage to facial musculature. The three basic designs of dialysis probe currently used, transcerebral, concentric, and U-shaped, are shown in figure **6.1.**

Microdialysis offers several advantages over other neurochemical techniques. The dialysis probe can reflect rapid changes in the extracellular neurotransmitter levels (Auerbach *et al.*, 1989). Probe implantation produces minimal tissue damage, it is a closed system producing clean samples which do not require lengthy preparation before analysis and it can be used in anaesthetised and freely moving animals to measure extracellular neurotransmitter levels. The probe is stereotaxically implanted into the specific brain region with reference to one of two specific points, Bregma and Lambda, on the surface of the rat skull (figure **6.1.**)

FIGURE 6.1. RAT SKULL AND DIFFERENT TYPES OF DIALYSIS PROBE. A. Top view of a rat skull showing the position of the two reference points, Bregma and lambda, used for stereotaxic implantation of microdialysis probes, adapted from Paxinos and Watson (1982). **B.** Original transdermal dialytrode, after Delgado *et al.*, (1972). **C.** Transcerebral and **D.** Concentric (upper) and U-shaped probes (lower). Redrawn from Di Chiara *et al.*, (1990).



Stereotaxic surgery uses 3D coordinates: bregma is a reference point for the anterior/posterior (A/P) plane and the lateral plane. Depth measurements can be taken from the intraaural line or the surface of the skull or dura. Lamda is not generally used as a stereotaxic reference point but coincidentally appears near the intraaural line.

Figure **6.1. D.** shows a commonly used microdialysis probe, the concentric probe developed by Tossman & Ungerstedt, (1986). The perfusion fluid enters the top end of the inner tube. It leaves the inner tube close to its lower end through two holes in the wall. The liquid then flows upward in the space between the dialysis membrane and the inner tube, where diffusion of substances from the tissue takes place. The perfusate leaves the probe via a small tube extending at a right angle to the probe. The pore size of the dialysis membrane determines the molecular-weight limit of the compounds entering the probe.

The main purpose of microdialysis is to study the composition of the brain interstitial space under various conditions. Because microdialysis does not obtain samples of the interstitial space, the concentrations of substances in the outflow solution are only a fraction of the true brain interstitial concentrations. Therefore, the microdialysis probe has to be calibrated before use *in vivo*. A description of the procedures usually carried out when using microdialysis follows.

Calibration: The term 'recovery' or relative recovery is defined as the ratio between the concentration of a particular substance in the outflow solution and the concentration of the same substance in the solution outside the probe (Ungerstedt *et al.*, 1982). Absolute recovery is the amount of a substance harvested in the outflow per unit time.

Recovery *in vitro* is usually measured as follows: the probe is continuously perfused at a constant flow rate and inserted into an aqueous solution of the substance at a known concentration. The perfusion fluid is identical to the medium outside the probe except for the substance of interest. The probe is perfused at least 30 min before samples are collected. Several samples are collected at fixed intervals (e.g. every 20 min). The substance concentration in the outflow is determined (e.g. HPLC), and the relative recovery is calculated as

$$\text{Recovery}_{in\ vitro} = C_{out} / C_i$$

where C_{out} is the substance concentration in the outflow and C_i is the substance concentration in the medium.

In vivo, the probe is implanted into the brain and perfused for 1 to 2 h before samples are collected manually or automatically (fraction collector). Samples are then

analysed immediately e.g. HPLC (Wages *et al.*, 1986) or frozen at -70°C and stored for later analysis.

Finally the true interstitial substance concentration is calculated as

$$C^*i = C^*out \,/\, \text{recovery } in\ vitro$$

where C^*i is the true interstitial concentration and C^*out is the substance concentration in the *in vivo* outflow solution.

The validity of these procedures rests on the assumption that conditions *in vitro* are equal to those *in vivo*. This is obviously not true, and even assuming that insertion of the probe into the brain does not change the tissue microenvironment, the following factors affect *in vivo* and *in vitro* recovery.

Perfusion flow rate: Perfusion flow rate is inversely related to relative recovery (Benveniste, 1989). However, the flux of substances across the dialysis membrane is not significantly influenced by perfusion rates above 2.5 µl/min (Zetterstrom *et al.*, 1982; Hamberger *et al.*, 1983; Ungerstedt, 1984). This is most likely caused by diffusion limitation and possibly by a positive hydrostatic pressure gradient across the membrane with higher flow rates. A rigid tube design can build up a positive hydrostatic pressure across the membrane at higher flow rates and cause a decrease of mass transport. At a given flow rate, the following additional factors have a significant influence on recovery.

Time after start of perfusion: Relative recovery has been determined to be time independent; initially, relative recovery is high but rapidly decreases. However this may be neglected after perfusion for 60 min, because it only constitutes a minor factor (<1% after 1 h). The problem is circumvented by delaying sample collection for at least 60 min.

Diffusion coefficients: The relative recovery of various substances measured is known to vary. This may be due to differences in molecular weight and, therefore, altered diffusion coefficients. Moreover, diffusion in a non-homogeneous medium is impeded by cell membranes, which gives rise to tortuosity - a prolongation of the diffusion pathway, as illustrated in figure **6.2.** For this reason, mass transport into the dialysis fibre is less *in vivo* compared with *in vitro*.

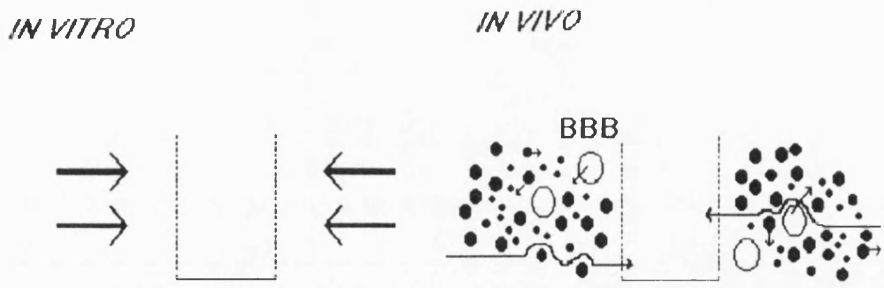


FIGURE 6.2. Diffusion in a tissue is impeded by cell membranes, which give rise to tortuosity - a prolongation of the diffusion pathway. Therefore mass transport into the microdialysis probe is less *in vivo* compared with *in vitro*. Note also that cells cannot be considered as inactive bodies. The brain interstitial substance concentration will depend on (a) transport across the blood brain barrier (BBB) and (b) cell (neurons and glial cell) metabolism. Figure redrawn from Benveniste, 1989.

Substance concentrations: Whereas relative recovery is independent of the outer substance concentration, absolute recovery is proportional to it (Hamberger *et al.*, 1983; Ungerstedt, 1984).

Substance interaction with the dialysis membrane: Membrane materials may interact with transported substances via unknown mechanisms and affect mass transport. For instance, recovery of acid metabolites is much higher than that of the parent amine, even for substances with similar diffusion coefficients (Ungerstedt, 1984).

Temperature: Diffusion coefficients increase 1-2% / °C and hence recovery at 37°C is considerably greater than at 22°C. This problem has been circumvented by several investigators by performing the *in vitro* test at 37°C (Zetterstrom *et al.*, 1982)

Membrane area: Relative and absolute recoveries increase in direct proportion to the area of the dialysis membrane area (Hamberger *et al.*, 1983; Ungerstedt, 1984)

A great many factors therefore affect the *in vivo* recovery of the substance of interest, and the correct determination of the absolute concentration of a particular substance e.g. neurotransmitter, require complex mathematical models, taking into account all of the above-mentioned factors. In practise, many of these variables are difficult, if not impossible to estimate, including: diffusion in the interstitial space; cellular uptake, exchange mechanisms, and metabolism; the diffusion characteristics for most substances.

It is worth bearing in mind that although simple in principle, brain dialysis is an invasive technique: it attempts to monitor the extracellular concentrations of transmitter

released from nerve terminals by inserting into the brain, a probe several orders of magnitude larger than the biological structures under study. The outer diameter of currently available probes (0.3-0.8 mm) is about a thousand times that of the synapses from which neurotransmitter release is to be monitored. Indeed, despite using the term 'microdialysis' brain probes are 10-100 times larger than other tools (micropipettes, microelectrodes) currently used by neuroscientists. A particular concern is that the blood-brain barrier (BBB) is intact during microdialysis to avoid contamination from the systemic circulation. Hamberger *et al.*, (1983) and Hamberger & Nystrom (1984) compared the concentration of amino acids in blood, cerebrospinal fluid (CSF) and tissue with concentrations in microdialysis perfusates. They found that interstitial amino acid concentrations calculated from the *in vitro* recovery were distinctly lower than those of blood and tissue but fitted remarkably well with CSF values. Administration of α-aminoisobutyrate, an inert, neutral amino acid which does not cross the BBB rapidly, demonstrated that the BBB was intact 30 min after insertion of the microdialysis probe but not <10 min after insertion (Benveniste *et al.*, 1984) though this will be affected by the probe size.

Another concern is that the environment surrounding the dialysis probe should be as representative as possible of normal, non-pathological, brain tissue. A histological examination of tissue reactions to the microdialysis probe was reported by Benveniste & Diemer (1987). Within the first 2 days, normal neuropil and only occasional haemorrhage surrounded the tube. On the third day, an astrocyte reaction was seen using an antiserum against glial fibrillary acidic protein. Hypertrophic astrocyte processes were observed within the spongius fibre wall. Fourteen days after the implantation, layers of reticulin-positive fibres surrounded the dialysis membrane. These chronic reactions are held to be unavoidable in the vicinity of both implants and lesions (Stensaas & Stensaas, 1976). .Obviously, the coating of the probe with inflammatory cells and connective tissue could change control diffusion characteristics of the tissue and fibre.

Changes in local cerebral glucose metabolism (LCMRglc) and blood flow (LCBF) are sensitive indicators of the tissue damage caused by implantation, and may reveal damage when brain tissue appears histologically normal. Consistent changes in both LCMRglc and LCBF within 3 h following the implantation of the probe, have been reported by Benveniste *et al.*, (1987). Localised regions of increased glucose metabolism and decreased flow were found in the hippocampus in close proximity to the fibre. Animals allowed to recover for 24h did not show these changes, but they exhibited a slight uniform decrease of LCBF. Changes of LCBF and LCMRglc could be due to the induction of a

spreading depression by the tissue trauma inflicted by insertion of the probe into the brain matter (Shinohara *et al.*, 1979). A spreading depression is characterised by arrest of spontaneous and evoked electrical activity, an increase in extracellular potassium concentrations, and a decrease of calcium content.

In summary, the implantation of the dialysis probe causes some disturbance in tissue metabolism, but this can be neglected at 24 h after implantation, because the dialysis technique is by then stable with respect to LCMRglc and LCBF, (though care should be taken to obtain samples before 2 days if possible, to avoid the adverse histological reactions mentioned above). Other problems related to disturbed cellular metabolism are perhaps obviated by sampling dialysates for 2-3 h to obtain stable baseline concentrations of various metabolites.

6.1.2. MICRODIALYSIS AND ACh RELEASE

The feasibility of measuring ACh by means of *in vivo* brain dialysis in freely moving rats requires a sensitive assay to detect the picomolar concentrations of the transmitter. Moreover, because of the problems of ACh degradation by cholinesterases (section **4.3.4.5.**) it is necessary to include a cholinesterase inhibitor such as neostigmine in the perfusing solution in order to measure basal ACh levels. The development of a sensitive HPLC assay facilitated the detection of the neurotransmitter in the mid 1980's (Damsma *et al.*, 1985), though radioimmunoassay has also been used. Two groups in 1987 (Damsma *et al.*, 1987; Consolo *et al.*, 1987) were among the first to demonstrate increased ACh release in the rat brain (striatum in both cases), in response to systemic injection of muscarinic drugs. Damsma *et al.*, (1987) demonstrated a significant increase of ACh (250%) 40 min after the injection of the antagonist atropine (10 μmol/kg, i.p.). In contrast no change in basal ACh release was reported after injecting the agonist oxotremorine (5 μmol/kg, i.p.). Consolo *et al.*, (1987) used the antagonist scopolamine (0.34 mg/kg, s.c.) and demonstrated an increased ACh release of approximately 200-250%, 20 to 40 min after injection; oxotremorine, on the other hand, caused a small decrease (60% of basal) in striatal ACh release, 20 min after injection.

In vivo studies involving ACh release have not been limited to muscarinic agonists or solely to studies of the striatum. Hutson *et al.*,(1991) initially verified the scopolamine studies of Consolo and Damsma in striatum, and found that a higher dose (1 mg/kg, i.p.) caused a large increase (960%) of ACh release. This was thought to be a centrally mediated

effect because repeating the study with N-methyl-scopolamine which has poor penetrance of the blood-brain-barrier, demonstrated a much smaller (177%) increased ACh release. Scopolamine (1 mg/kg, i.p.) also mediated a very large increase (2350%) in hippocampal ACh release, 40-60 minutes following injection. The authors concluded that the results were consistent with an inhibitory action upon the hippocampal muscarinic autoreceptors. Hutson *et al.*, (1991) went on to demonstrate increased ACh release (180-200%) in response to thyrotropin releasing hormone (TRH), which has been shown to activate the cholinergic septo-hippocampal pathway (Kalivas & Horita, 1979). The actions of TRH and various metabolically stable analogues were found to be specific to the hippocampus, and had no effect upon striatal ACh release, but the authors were careful to point out that TRH also stimulates noradrenaline release, so it was conceivable that increased hippocampal ACh was in indirect effect of noradrenergic stimulation. This raises an important issue, namely that following the systemic injection of drugs, a variety of brain transmitter systems in one or more brain regions are likely to be affected. It is important therefore, not to over-interpret the results obtained in these studies. For example, while TRH may directly stimulate the septo-hippocampal cholinergic system, it is not possible to rule out indirect noradrenergic effects without conducting more experiments. This is an issue which will be discussed in relation to the present study in section **6.4.**

6.1.2.1. Hippocampal ACh Release in Response to Nicotinic Drugs

In 1989 Toide & Arima, (1989), reported the use of microdialysis to monitor effects of both muscarinic and nicotinic drugs on extracellular levels of ACh in the rat hippocampus. Administration of scopolamine (0.5 mg/kg s.c.) caused a profound increase in the release of ACh (>600% increase over basal release) in the hippocampus, in agreement with the studies outlined above. Systemic administration of nicotine (0.5 mg/kg s.c.) also enhanced hippocampal ACh release, at 30 min and 90 min after administration. This nicotine-evoked ACh release was quite modest but significant (160%), which could be consistent with stimulation of presynaptic nAChRs, was also seen in the frontal cortex and the striatum, and in each region the release was also biphasic in nature. Toide & Arima attributed this phenomenon, whereby ACh release was enhanced, fell back to basal levels, then was enhanced once again (without further injection of agonist), to 'receptor desensitisation'.

Since the focus of this project was to study how the presynaptic nAChRs modulate ACh release in the hippocampus. So far, the *in vitro* studies have demonstrated that agonists enhance [^{3}H]ACh release from synaptosomes, and it would be interesting to demonstrate that this was a relevant physiological phenomenon, i.e. nicotine was able to enhance hippocampal ACh *in vivo*. Hence the initial studies of Toide & Arima (1989) were repeated, with a view to characterising any nicotine-evoked response in terms of concentration dependency. Using a nicotinic antagonist, it was also hoped that any response could be shown to be mediated by nAChRs, just as the *in vitro* responses were shown to be nAChR-mediated (section **4.3.2.2**).

6.2. METHODS

The protocols for the care of the experimental animals, the surgical procedures, the microdialysis perfusion protocol and the analysis of samples by HPLC, are all detailed in Chapter 2. Microdialysis was always carried out using conscious, freely moving rats over a period of five hours.

6.3. RESULTS

6.3.1. EFFECTS OF SYSTEMIC ADMINISTRATION OF NICOTINE

To allow the establishment of a stable baseline samples were collected for two hours before the rats were injected with nicotine.. Figure **6.3.** shows the changes in the time course of ACh in dialysates of rat hippocampus following administration of (-)nicotine (0.4 or 0.6 mg/kg s.c. in the right flank). The lower dose of nicotine (0.4 mg/kg) increased ACh release (about 180%) in hippocampus transiently but significantly, 40 min after its administration. The ACh level then reverted to the control value but subsequently increased (130-140%) again, during the period 100- 160 min after administration, the second increase did not reach statistical significance. The higher dose of nicotine (0.6 mg/kg) produced smaller increases in hippocampal ACh levels: a significant increase was noted at 60 min (approximately 150%), after which there was a return to basal release levels, followed by a second increase (non-significant; 130-140%) at 160 min.

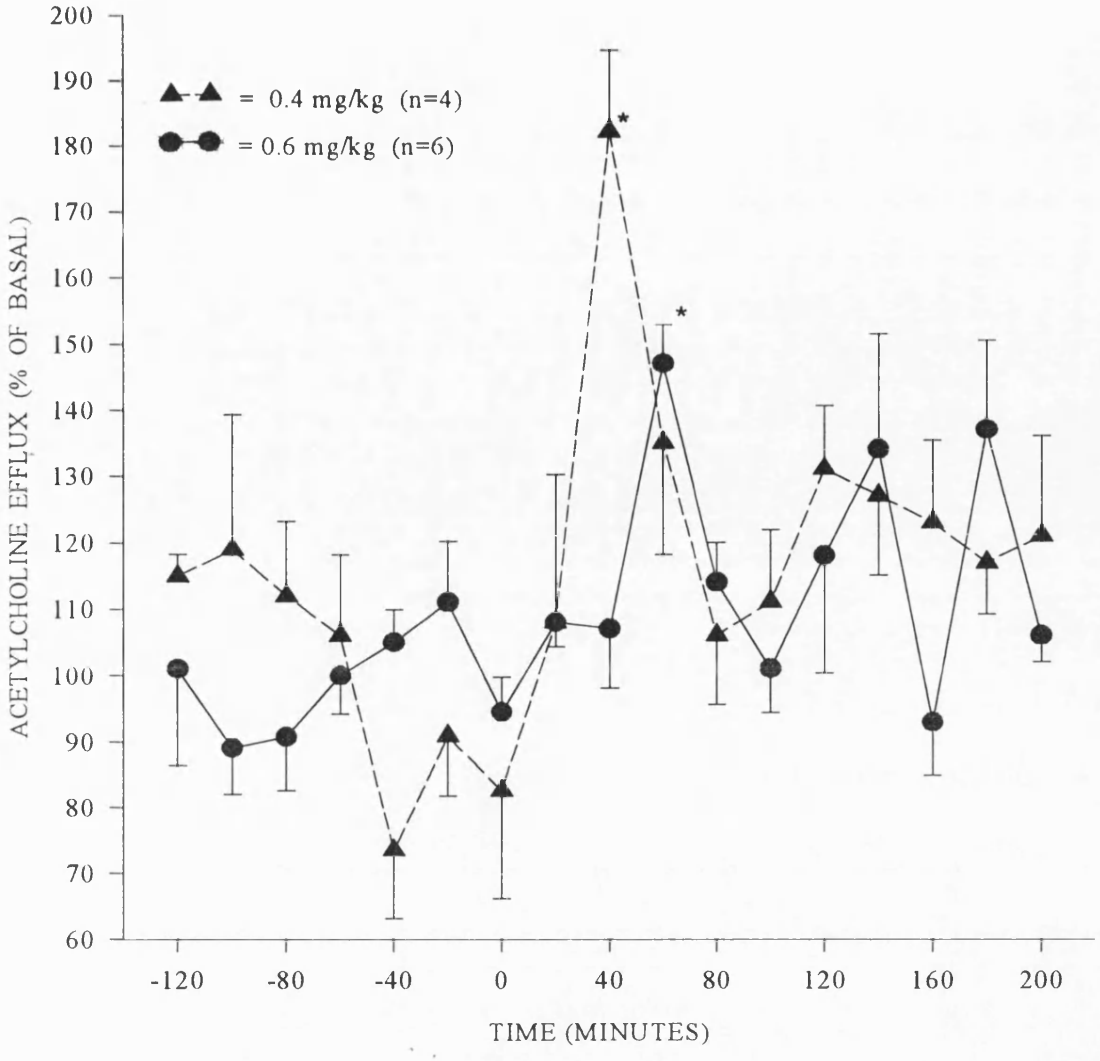


FIGURE 6.3. NICOTINE-EVOKED, *IN VIVO* RELEASE OF HIPPOCAMPAL ACETYLCHOLINE. Values are mean ± SEM release as a percentage of pre-injection basal values. * = significantly different from basal release at $p<0.05$. Basal = 53 fmol/min.

6.3.2. EFFECTS OF SYSTEMIC ADMINISTRATION OF MECAMYLAMINE

In order to further characterise the *in vivo* response to nicotine, it was necessary to demonstrate that the observed increases in hippocampal ACh levels were mediated by nAChRs, hence the use of a nicotinic antagonist. Control experiments were therefore initially undertaken using the antagonist mecamylamine alone.

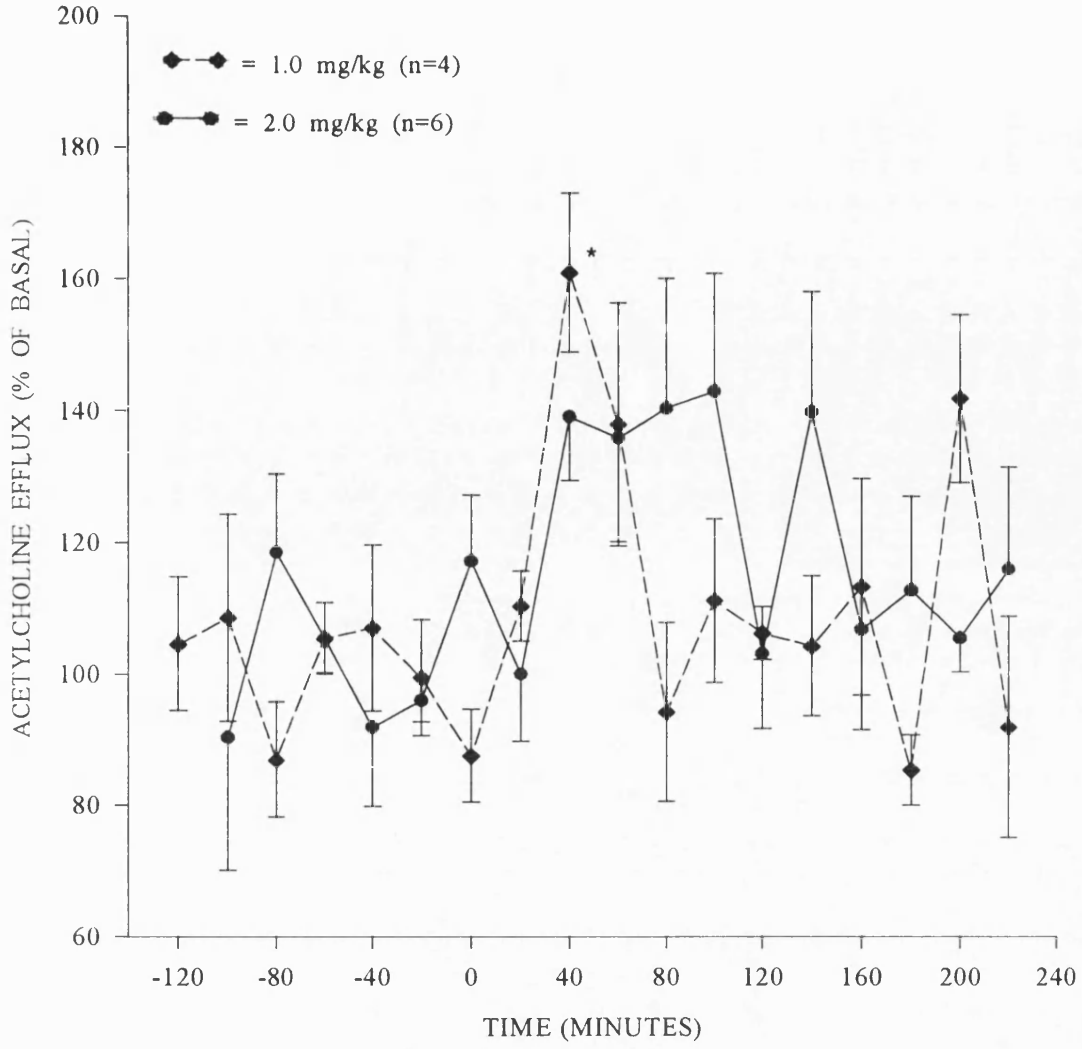


FIGURE 6.4. MECAMYLAMINE-EVOKED, *IN VIVO* RELEASE OF HIPPOCAMPAL ACETYLCHOLINE. Values are mean ± SEM release as a percentage of pre-injection basal values. * = Significantly different from basal release at $p<0.05$. Basal = 65 fmol/min.

Figure **6.4.** shows the results of administration of mecamylamine (1.0 or 2.0 mg/kg, i.p.) at time zero. The dose of 1.0 mg/kg mecamylamine produced a transient but significant ($p<0.05$) increase (161%) in ACh at 40 min, returning to baseline levels after 80 min, before increasing modestly (not significant; 140%) after 200 min. This pattern of biphasic release mimicked that seen with 0.6 mg/kg nicotine, albeit over a different time scale. The higher dose of 2 mg/kg induced behavioural signs in the experimental animals (prostrate body position and shallow breathing), and produced a sustained increase in ACh release (130-140%), for 2 h post-injection. This increase never reached statistical significance because of the large variability in response between animals, indicated by the standard error bars in figure **6.4.**

6.4. DISCUSSION

6.4.1. NICOTINE ADMINISTRATION

As mentioned in section **6.1.2.** a single research paper has been published (Toide & Arima, 1989) detailing the effects of nicotinic agonists on ACh levels in the rat hippocampus, cortex and striatum. The report described a transient 160% increase in hippocampal ACh release following administration of nicotine (0.5 mg/kg, s.c.). this initial rise was followed an hour later by a more modest rise of 130%, a pattern similar to the results reported in section **6.3.** Toide & Arima stated that this biphasic pattern of release could 'presumably be attributed the desensitisation effect', whereby they postulated that 'a prolonged exposure to nicotine could induce desensitisation'.

Results from the superfusion studies (chapter 6) indicate that the hippocampal nAChR undergo rapid desensitisation within seconds/minutes. The fact that nicotine was injected subcutaneously in the microdialysis protocol means that the drug enters the brain over a period of time, rather than as a single discrete bolus. Benowitz *et al.*, (1990) reviewed the pharmacodynamics of (-)nicotine: in a rat of between 170 and 340 g (the rats in the present study were always 250-350 g), at a dose of 0.4 mg/kg, the terminal half life of the nicotine is approximately 55 min. Exposure of the nAChRs to a desensitising concentration of agonist would result in a burst of elevated transmitter release, followed by receptor desensitisation - resulting in a return to basal release, followed by a refractory period in which the receptors could be expected to slowly resensitise, as the concentration of nicotine in the microenvironment of the synapse falls. This sequence of events would explain the pattern of ACh change induced by nicotine in figure **6.3.** The half life for the higher dose of nicotine used in this study (0.6 mg/kg), could be expected to be slightly longer than 55 min, and could explain the slightly different temporal pattern of ACh elevation. What is not immediately obvious is the reason for the second, modest rise of ACh occurring approximately 1 h after the initial phase of release. If the nAChRs have resensitised there is a possibility that there was still a sufficient level of nicotine in the synaptic microenvironment to trigger receptor-mediated release of ACh. Since the half life of the agonist is of the order of 1 h, significant levels of nicotine could be present 120 min after the injection, taking into account the fact that nicotine was likely to arrive in the brain some minutes after the injection, build up in concentration, then fall as the rate of metabolism exceeds the rate of delivery.

6.4.2. MECAMYLAMINE ADMINISTRATION

Mecamylamine seemed to be the ideal antagonist with which to probe whether the observed effects of nicotine were nAChR-mediated: it is routinely used to block the biochemical (Rapier *et al.*, 1990) and behavioural effects (Levin, 1992; Stolerman *et al.*, 1973) of nicotine, and readily penetrates the CNS. The experiments with mecamylamine alone were designed as controls to demonstrate that the antagonist had no inherent effect on ACh release in the hippocampus, prior to experiments in which nicotine and mecamylamine were to be co-administered. Since the systemic delivery of the antagonist alone provoked an increase in hippocampal ACh levels, co-administration would not reveal whether the nicotine-evoked release was mediated via the nAChRs.

One possible explanation for the ability of mecamylamine to increase ACh levels in the hippocampus is that it is acting in a manner akin to the NMDA antagonist dizocilpine (MK-801), which also produces an increase in hippocampal ACh release when injected systemically (0.3 mg/kg, i.p.; Hutson, 1993 personal communication). It is unclear whether this action of MK-801 is mediated via the hippocampal nAChRs or is an indirect effect following antagonism of hippocampal NMDA receptors. Alternatively, systemic MK-801 may inhibit dopamine release from the ventral tegmental area which in turn my activate the septal-hippocampal cholinergic pathway and result in increased hippocampal ACh. In studies on nicotine-evoked [^{3}H]GABA release from hippocampal synaptosomes, high concentrations of MK-801 have been shown to directly interact with neuronal nAChRs, possibly by acting as a nicotinic channel blocker (Wonnacott *et al.*, 1988).

The interaction between mecamylamine and MK-801 is complex: mecamylamine and MK-801 are both able to inhibit nicotine-induced locomotor activity in rats (Clarke & Kumar, 1983; Shoaib & Stolerman, 1992) and it has been reported that mecamylamine inhibited [^{3}H]MK-801 binding to rat brain membranes, and increased [^{3}H]MK-801 dissociation rates, suggesting that it was acting at the MK-801 binding sites (Reynolds & Miller, 1988). Confirmation that mecamylamine is a selective non-competitive antagonist of NMDA receptors was reported by O'Dell & Christiansen (1988). The possibility that mecamylamine increased hippocampal ACh release in the present study via interactions with hippocampal NMDA receptors, rules out its further use to characterise the nicotine-evoked ACh release.

It was unfortunate that time constraints prematurely ended the microdialysis experiments, especially at such an inconclusive stage. From the results of the study, nicotine can be said to increase hippocampal ACh levels, though it is not possible to state with any certainty whether this is a peripherally- or centrally-mediated effect. One approach to distinguish the two possibilities would be to use a peripheral nicotinic antagonist such as hexamethonium, which has poor CNS penetrance and which would differentiate between the peripheral and central actions of systemically-administered nicotine (Rosecrans, 1987). Another approach is to administer nicotine directly into the hippocampus either by injection (icv) or via the microdialysis probe itself, which ideally, would obviate the need to use peripheral antagonists. The problem of demonstrating that nAChRs mediate the nicotine-evoked hippocampal ACh release, could be resolved using an alternative nicotinic antagonist such as DHβE either systemically or, like nicotine, via the dialysis probe. It would obviously be necessary to initially ascertain whether DHβE itself affected hippocampal ACh release.

One problem with studying the nicotinic enhancement of ACh release in the brain is that the nicotine-evoked responses were small. The peak response to nicotine in this study and that of Toide & Arima (1989) was an increase of only 150-180% of basal ACh release, compared to the peak muscarinic antagonist-evoked release of ACh which was approximately 2000% (Toide & Arima, 1989; Hutson *et al.*, 1991). This would be less of a problem except basal ACh release was found to be quite variable. Basal release was calculated as the mean ACh release over the two hour period immediately prior to drug treatment. Rats tended to vary considerably with respect to one another in terms of mean basal ACh release values (range = 32-98 fmol/min), and to further complicate interpretation, the basal release for individual rats, whilst usually remaining fairly steady between 80-120% of the mean, was also subject to occasional 'spikes' - increases of more than 140% for isolated time points. This variation resulted in large standard errors even for basal release, so drug effects were occasionally masked, or, obvious peaks in the ACh release profile struggled to reach significance. Another factor confounding interpretation of the results was the continuous presence of neostigmine in the aCSF used to perfuse the probe: the cholinesterase inhibitor was a prerequisite for detection of basal ACh levels. Necessarily, neostigmine artificially enhanced ACh levels in the local environment surrounding the probe and from the results in chapter 4, it is possible that these increased ACh concentrations continuously stimulate the hippocampal autoreceptors which regulate ACh release. The pattern of evoked ACh release in figure **6.3.** may be complicated by autoreceptor desensitisation induced by neostigmine-enhanced ACh levels.

In summary, systemic injection of two concentrations of nicotine evoked hippocampal ACh release in freely moving rats, though this release was not dose-dependent. Initial attempts to characterise the evoked release using the nicotinic antagonist mecamylamine were frustrated by the ability of the antagonist to evoke ACh release in its own right. Further dialysis work could address the *in vivo* significance of the hippocampal nicotinic autoreceptors (discussed in section **7.5.**).

CHAPTER 7. CONCLUSIONS

The pentameric structure of the neuronal nAChR and the considerable molecular diversity in subunits provides the possibility of a large number of nAChR subtypes which may be anticipated to subserve a variety of functions within the CNS. This study was undertaken to develop a better understanding of the nicotinic presynaptic autoreceptors responsible for modulation of ACh release in the rat hippocampus. In addition to the basic goal of further elucidating the pharmacology of these nAChRs, with the ultimate aim of identifying the subtype identity, the study was relevant to the ongoing search for new therapeutic targets for dementing diseases such as AD.

7.1. nAChRs AND AD

AD is associated with a loss of learning and memory abilities, attentional deficits, anxiety, agitation and depression (Perry, *et al.*, 1978). Other impaired brain functions include reductions in cerebral blood flow, cerebral glucose utilisation and abnormal EEG (Petit *et al.*, 1993). Topographically these impaired brain functions correspond to the loss of the cholinergic innervation arising from the basal forebrain (Coyle *et al.*, 1983) and to substantial reductions in neuronal nAChRs (Whitehouse, *et al.*, 1981; 1986; Perry *et al.*, 1986).

Although the prevailing dogma suggests that mAChRs mediate the primary effects of central cholinergic transmission on cognitive performance (Bartus *et al.*, 1982; 1985) and cerebral vasodilation (Lee, 1982; Pinard, 1989; Hamel & Estrada, 1989), replacement therapy targeting mAChRs has not been a fruitful approach to the amelioration of AD symptomatology (Arneric & Williams, 1993).

Neuronal nAChRs in the CNS represent an expanding area of potential therapeutic opportunity driven by new findings in the molecular biology of the system (Changeux *et al.*, 1992; Deneris *et al.*, 1991; Sargent, 1993). The pharmacological properties and physiological function of these newly identified molecular targets remain, to a large extent, unknown primarily due to a lack of potent and selective pharmacological probes. Nonetheless, alterations in the activity of the acetylcholine-gated ion channels have been implicated in a number of CNS disorders including AD (Arneric & Williams, 1994). Preliminary clinical data indicate that acutely administered nicotine, the prototype agonist

for nAChRs may be beneficial for the treatment of the deficits in attention and information processing associated with AD (Wesnes & Warburton, 1984; Newhouse *et al.*, 1988; Sahakian *et al.*, 1989; Jones *et al.*, 1992).

Extensive evidence exists to indicate that activation of nAChRs improves cognitive performance and improves cerebral functions in experimental animals and normal humans. Nicotine enhances cognitive function in normal rats (Levin *et al.*, 1990; Levin, 1992) and attenuates memory deficits produced by destruction of cholinergic input to the cortex and hippocampus (Tilson *et al.*, 1988; Decker *et al.*, 1992; Hodges *et al.*, 1992), an effect shared by some other nAChR agonists (Decker *et al.*, 1993; Meyer *et al.*, 1994). In addition, nicotine improves short-term memory performance in both young and aged monkeys (Elrod *et al.*, 1988; Buccafusco & Jackson, 1991). The involvement of nicotinic neurotransmission in cognitive functions processes is further substantiated by observed deficits in cognitive performance after administration of mecamylamine, a nAChR channel blocker, to rodents (Levin *et al.*, 1987), monkeys (Elrod *et al.*, 1988) and humans (Newhouse *et al.*, 1992). Moreover the characteristic cerebral cortical blood flow abnormality associated with AD reflects nAChRs deficits. Specifically, it has been demonstrated that mecamylamine, but not the muscarinic antagonist, scopolamine, reduces resting cerebral blood flow in the parietotemporal cortex of humans (Gitelman & Prohovnik, 1992), the area most consistently implicated in functional brain imaging of AD patients (Heiss *et al.*, 1990). Reduced nicotinically mediated cerebral blood flow responses would thus be consistent with the loss of nAChRs reported in several cortical regions using various labelling techniques (Araujo *et al.*, 1988; Aubert *et al.*, 1992) as well as a report suggesting a loss of basal forebrain nAChR population in AD (Shimihama *et al.*, 1986).

7.2. AGONIST THERAPY

Nicotine however, has limited utility as a therapeutic agent for AD because of its dose-limiting side effects in humans, which are primarily gastrointestinal (e.g. nausea, abdominal pain) and cardiac (e.g. increased catecholamine resulting in tachycardia, peripheral vasoconstriction and elevated blood pressure) in nature. In an aged patient population these latter effects may result in more serious complications (Benowitz, 1992), especially in patients with pre-existing arrhythmias or angina pectoris. Compounds that selectively interact with subtypes of nAChRs to normalise CNS functions governed by this receptor family may, therefore, lead to more effective therapeutic agents.

The potential for therapeutic use of nicotinic agonists is further complicated by the phenomenon of nAChR desensitisation/inactivation. The results presented in chapter 4 demonstrated "bell-shaped" dose response curves for agonist-evoked [^{3}H]ACh release. Nicotinic responses measured biochemically over a similar timecourse (40 s to a few minutes) typically result in inverted-"U"-dose responses (Marley, 1988; Boyd, 1987), as seen for [^{3}H]ACh release here. Indeed this is a common phenomenon in pharmacology (Pliska, 1994), attributed to desensitisation of some step in the process being measured; in this case desensitisation or inactivation of the nAChR itself is likely (Marley, 1988). Because of this narrow concentration range over which nicotine increases ACh release, therapeutic administration of nicotine would require careful dosing. Due to the considerable individual variability in nicotine metabolism (Benowitz, *et al.*, 1982) the dose of drug would have to be titrated individually in each patient; ideally the dose would cause maximal transmitter release and minimal nAChR desensitisation.

The propensity of nAChRs to desensitise, is in addition to the side-effects such as nausea and emesis associated with nicotine administration, detailed above. A potential therapeutic outcome of developing compounds that selectively interact with nAChR is that they do not necessarily elicit a side-effect profile like nicotine. Behavioural testing of the agonist ABT 418, have indicated that compared with nicotine, ABT 418 is significantly less potent in producing hypothermia, acute toxicity, overt seizure activity, altered resting cerebral blood flow and reducing locomotor activity (Decker *et al.*, 1994). As such the agonist ABT 418 may represent the first of new class of compounds that selectively activate neuronal nAChRs without eliciting the dose-limiting side effects typically observed with nicotine, though it remains to be seen whether nAChR desensitisation observed *in vitro* would be reflected *in vivo*. The potential for developing nAChR ligands for use in treating AD (not to mention smoking cessation, anxiety, and depression) appears high, providing a challenge and emerging therapeutic opportunity. Table **7.1** outlines the various lead compounds presently in development.

COMPANY	COMPOUND	INDICATION	STATUS
Ciba, Warner-Lambert	Nicotine patches	Smoking cessation AD?	Launched
DynoGen	Lobeline, 7 day injectable	Smoking cessation	Phase III
Abbott	ABT-418 patch formulation	AD/smoking cessation	Phase I
Taiho/Univ. Florida, Gainesville	GTS-21/anabaseine analogues	AD	Preclinical (patients)
R.J. Reynolds	RJR 1401	AD neurodegeneration	Preclinical (patients)
Philip Morris	Nicotine analogues	Unknown	Preclinical
SIBIA	Patent application for human nAChRs	AD	Preclinical
Lilly	Iso-arecolone analogues	AD	Preclinical (patients)
Merck	Anatoxin analogues	AD	Preclinical (patients)

TABLE 7.1: POTENTIAL THERAPEUTIC DRUGS BASED ON NICOTINE AGONISTS. Adapted from Williams *et al.*, 1994.

7.3. HIPPOCAMPAL nAChR AUTORECEPTOR SUBTYPE

Attempts to delineate the nAChR subtype(s) responsible for the modulation of hippocampal ACh release were not entirely successful. The paucity of ligands selective for the various putative nAChR subtypes effectively limits the investigation of native nAChRs to pharmacological comparisons with heterologously expressed nAChRs (cell lines, oocytes) and the use of the few selective ligands (αBgt, MLA). Nevertheless significant progress was made. The reported lack of inhibition by the α7-selective toxin αBgt (Wonnacott *et al.*, 1989) was confirmed using another α7-selective antagonist - MLA, indicating that α7-containing nAChRs cannot account for the observed pattern of transmitter release. The finding that cytisine was a full agonist in the hippocampal preparation seemed to rule out β2-containing nAChRs (Luetje & Patrick, 1991), the agonist rank-order of potency of:

$$\text{ANTX} > \text{nicotine} \approx \text{cytisine} \approx \text{ACh} > \text{iso-arecolone}$$

matched none of the oocyte data in the literature however, in which cytisine was found to be a weak agonist and much less potent than nicotine on β2-containing nAChRs, and conversely in β4-receptors cytisine was a more potent agonist than either nicotine or ACh (reviewed in Zwart *et al.*, 1994). It is clear that the oocyte data do not tell the whole story though: the subtype identity of native neuronal nAChRs is unclear, agonist potency rank-orders of native nAChR-mediated currents in rat medial habenula and interpeduncular nucleus for example, also do not resemble any of the rank-orders of nAChRs expressed in oocytes (Mulle *et al.*, 1991). This is not surprising since it has been shown that neurons may express nAChRs composed of more than two types of subunits and more than one nAChR subtype (Vernallis, *et al.*, 1993). Both the expression of more complex subunit assemblies and the co-expression of more than one type of nAChR might give rise to a mixed pharmacological profile.

A comparison of the agonist potency rank-order and the EC_{50} values in the present study with data from $^{86}Rb^{+}$-flux experiments using chick α4β2 nAChRs stably expressed in mouse M10 fibroblasts (Stephens, 1994; section **4.4.1.**) was instructive. Cytisine was a full agonist at chick β2 subtype-containing receptors. Moreover, a comparison of the agonist EC_{50} values obtained in the two systems is instructive: despite species differences (chick vs. Rat nAChR) and large differences in the two methodologies, the values compare reasonably well and the α4β2 subtype remains a plausible candidate for the rat hippocampal autoreceptor. The lack of availability of selective ligands, especially the α3-selective toxin neuronal Bgt, have frustrated the further delineation of the possible nAChR subtype(s) located presynaptically in the hippocampus.

Defining the hippocampal autoreceptor subtype(s) is certainly an area in which the present work could develop. It would seem that as far as the hippocampal synaptosome preparation is concerned, there are limited immediate opportunities: development awaits advances in molecular biology, for example the expression of further defined nAChRs in model systems such as the chick α4β2 nAChRs in M10 cells, or natural product/synthetic chemistry to provide novel, selective nicotinic ligands.

7.4. nAChRs AND SP

SP did not appear to interact with the agonist binding site on the hippocampal nAChRs, changing neither the affinity of the nAChRs for the ligand [^{3}H]nicotine, nor the apparent density of hippocampal [^{3}H]nicotine binding sites. While SP had previously been

shown to be a competitive antagonist of nicotinic agonist binding using nAChR-enriched *Torpedo* membranes, this effect was thought to be dependent on the δ and to a lesser extent the γ subunit of the *Torpedo* nAChR, and if this were the case, the hippocampal nAChRs presumably lacking both δ and γ subunits, would not be expected to display SP antagonist sensitivity. SP did however appear to modulate the nicotine-evoked release of [^{3}H]ACh in a complex manner consistent with a possible enhancement of agonist-induced desensitisation/inhibition of recovery from desensitisation, counterpointed by an apparent facilitatory action of SP on the hippocampal synaptosomes, causing [^{3}H]ACh release. The presence of hippocampal SP/NK1 receptors, demonstrated by binding experiments with the ligand [^{3}H][Sar^9,Met(O$_2$)^{11}]SP provide an indirect pathway by which SP may effect nAChR modulation, involving the stimulation of second messenger systems, involving PI turnover and PKC activation (sections **5.4.3.** and **5.4.4.**).

7.5. MICRODIALYSIS

The microdialysis study, carried out over three months at Merck, Sharp & Dohme, was successful in that increased hippocampal ACh release was demonstrated in response to systemic injection of nicotine, agreeing with the literature (section **6.4.1.**). Most frustrating however was the demonstration of increased ACh release in response to the antagonist mecamylamine, casting doubt on the specificity of the nicotine-evoked results. This work has good potential for development and would complement the *in vitro* studies well. Ultimately, the synaptosome superfusion system is only useful as a paradigm if it is shown to model the *in vivo* events in the rat hippocampus, something which could be evaluated by complementary *in vivo* work. The *in vivo* significance of the hippocampal nicotinic autoreceptors has not been satisfactorily demonstrated to date, but the microdialysis technique has the potential to delineate the role(s) of these receptors in a manner which the *in vitro* studies cannot address. For example if a suitable lesioning agent were found that produced 70-80% depletions in ChAT and therefore more closely modelled the cholinergic deficit in AD (see appendix), it would be possible to directly assay the functional activity of the remaining hippocampal autoreceptors by infusing nicotine or other agonists down the microdialysis probe, and studying the release of ACh, therefore estimating the usefulness of agonist therapy at an early stage. Such studies using conscious, freely moving rats could be carried out simultaneously with behavioural testing to determine not only the biochemical but any cognitive-enhancing effects of nicotinic agonists.

7.6. EXCITOTOXIC LESIONS

Finally, of all the aims outlined in the introduction (section **1.8.**), the work involving lesioned animals remains the least developed. Two studies showed that it was possible to produce measurable albeit small ChAT depletions in the cortex and hippocampus using excitotoxic acids to lesion the basal forebrain, MSA and nBM areas. These studies set out to demonstrate that degeneration of the cholinergic innervation from these areas resulted in reductions in the binding density of [^{3}H]nicotine in the hippocampus and cortex, and hence the presence of high -affinity presynaptic [^{3}H]nicotine binding sites. The main problem was not the design of the experiments but the fact that small lesions (20% ChAT depletion) were produced, which made the successful detection of a correspondingly small reduction in [^{3}H]nicotine binding sites difficult, especially when only a proportion of the total sites are on cholinergic terminals and therefore subject to the lesion. The first step to developing this lesion work would be the demonstration of a lesion that was relevant to the cholinergic pathology in AD i.e. of the order of 70-80%. This would hopefully ease the problem of detecting small changes in [^{3}H]nicotine binding density. Once such a lesion could be reliably reproduced, numerous avenues are open for functional studies. As outlined above, it would be interesting to produce a pathologically relevant lesion, then carry out superfusion studies and ultimately microdialysis/behavioural experiments to compare the *in vitro* and *in vivo* effects of nicotinic drugs in both the healthy rat (the present work) and an animal model of cholinergic dysfunction.

In conclusion, the nicotinic stimulation of ACh release from rat hippocampus, defined in this thesis offers hope for therapeutic intervention in AD, although further work is required to fully define the nicotinic receptor subtype(s) involved and its *in vivo* action.

INTRODUCTION

As outlined in section **1.4.1.2.** excitotoxic lesions of the rat basal forebrain produce profound deficits in performance on a wide variety of tasks involving discrimination learning and memory. These observations have been widely taken to reflect damage of cholinergic projections from the nBM and the MSA to the neocortex and the hippocampus, respectively. If a sufficiently large proportion of the high affinity [^{3}H]nicotine binding sites (by implication $\alpha4\beta2$ nAChRs) are located presynaptically on cholinergic terminals, then lesions damaging the cholinergic innervation of the hippocampus or cortex would be expected to measurably decrease the [^{3}H]nicotine binding density in these areas. This was the rationale behind the lesion studies detailed in this section.

STUDY 1: INSTITUTE OF PSYCHIATRY

In a study set up with Dr. Helen Hodges of the Institute of Psychiatry, four groups of eight rats received bilateral MSA-nBM lesions with either ibotenic acid, quisqualic acid, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), or saline (sham lesioned to act as controls). These animals were allowed three months to recover from the joint bilateral MSA-nBM lesions before behavioural testing was carried out at the Institute, after which the animals were sacrificed and the frontal cortex (FC) and the hippocampus (HP) dissected out and sent in dry ice to Bath University. These brain regions were assayed for [^{3}H]nicotine binding (section **2.2.1.**), and the results compared with data from ChAT assays, carried out at the Institute of Psychiatry.

RESULTS

The results of the [^{3}H]nicotine binding assays are presented in the following table.

	CONTROL	IBOTENATE	QUISQUALATE	AMPA
CR	42.9 ± 3.6	45.7 ± 2.6	40.7 ± 7.0	34.9 ± 4.5
CL	42.5 ± 8.6	48.0 ± 6.2	49.7 ± 7.6	37.4 ± 3.2
HP	29.6 ± 3.8	34.9 ± 4.5	33.5 ± 4.3	32.6 ± 3.0

TABLE A.1: [^{3}H]NICOTINE BINDING IN THREE BRAIN REGIONS FOLLOWING EXCITOTOXIC LESIONS. Values are mean fmol [^{3}H]nicotine bound/mg protein ± SEM, n=8. CR = right frontal cortex, CL = left frontal cortex, HP = hippocampus.

Statistical analysis of these results using a paired Student's t-test, comparing each of the lesioned groups with the mean control binding in each brain area, showed no statistical differences for any of the lesioning agents at the $p<0.05$ level of significance.

The results of the ChAT assays, performed at the Institute were:

	CONTROL	IBOTENATE	QUISQUALATE	AMPA
FC	224 ± 10	171 ± 9	188 ± 9	140 ± 10
	(100%)	(76%)*	(84%)*	(62%)*
HP	231 ± 14	243 ± 10	227 ± 12	231 ± 9
	(100%)	(105%)	(98%)	(100%)

TABLE A.2: ChAT ACTIVITY IN CORTEX AND HIPPOCAMPUS FOLLOWING EXCITOTOXIC LESIONS. Values are mean pmol [^{3}H]ACh produced/min/mg protein ± SEM (% of control). Left and right cortices were combined. FC = frontal cortex. * = significantly different from control at $p<0.05$.

There were no significant differences between any of the lesions and the control group in the hippocampus, but in the frontal cortex each of the lesions produced a significant depletion of ChAT activity.

DISCUSSION

With regard to the hippocampal ChAT and [^{3}H]nicotine binding data, it is difficult to draw meaningful conclusions. In the absence of a demonstrable cholinergic lesion, i.e. significant ChAT depletion, the lack of change in [^{3}H]nicotine binding is unsurprising. On the other hand, each of the lesioning agents produced a significant ChAT depletion in the frontal cortex with no significant decrease in [^{3}H]nicotine binding sites. Since the lesioning agents were injected into the nBM, source of the neocortical cholinergic innervation, and a depletion in ChAT activity implies a degeneration of the neurons, why was there no corresponding decrease in cortical [^{3}H]nicotine binding? This result is not unique in the literature, several groups have reported lesions producing ChAT deficits in the

hippocampus or cortex with no corresponding decrease in [^{3}H]nicotinic agonist binding e.g. Vige & Briley (1988), who used ibotenic acid to lesion the nBM and subsequently found decreases in ChAT activity in the frontoparietal cortex, but no change in [^{3}H]nicotine binding.

The rats in the present study had three months to recover and were then trained for the behavioural testing, before sacrifice. The observed decreases in ChAT activity indicate that with each of the lesioning agents, between 60-80% of the neurons were spared and it is a possibility that during the recovery period there was a compensation by surrounding, or postsynaptic neurons leading to a recovery in nAChR number. Similarly the behavioural testing may have led to an upregulation of nAChRs. Dunnett *et al.*, (1991) reported that quisqualate lesions of the nBM producing 50% reductions in cortical ChAT activity, had marginal effects on a variety of behavioural tasks including standard tests for 'cognitive' deficits such as disrupted spatial navigation in the Morris water-maze, and delayed matching-to-position tasks. The same authors also reported reductions of more than 70% of neocortical ChAT activity.

Another possible reason for not demonstrating decreases in [^{3}H]nicotine binding despite decreases in ChAT, requires a consideration of the proportion of [^{3}H]nicotine binding sites that are located on cholinergic neurons. Since nicotine has been shown to interact with a variety of other transmitter systems including dopaminergic, serotonergic, GABAergic, it is possible that the proportion of presynaptic nAChRs on the cholinergic terminals affected by the excitotoxic lesions, is relatively small. Also, the ChAT data show that only approximately 20% of the cholinergic terminals were affected, hence the actual decrease in [^{3}H]nicotine binding sites may be too subtle to detect.

STUDY 2: MERCK SHARP & DOHME

Following the lesion studies at the Institute of Psychiatry, it was decided to set up some MSA-lesioned rats (quisqualate; Hodges *et al.*, 1991) at the industrial sponsor, Merck Sharp & Dohme (MSD). Dr Pete Hutson kindly performed the surgery and looked after the post-operative welfare of the animals (4 lesioned, 4 controls), in accordance with Home Office guidelines. Three months after the surgery the animals were sacrificed on site, and the hippocampi dissected out, by the author. To determine the success of the lesions, ChAT activity was assayed, and [^{3}H]nicotine binding was investigated. Also examined was the uptake of [^{3}H]Ch by Percoll F4 synaptosomes prepared from the lesioned and control

rats. This parameter has been the standard index of synaptosomal viability used in the superfusion experiments, and it was hoped that it would provide insights into the integrity of the lesioned tissue. Following partial destruction of the extrinsic cholinergic innervation of the hippocampus, it might be expected that there would be fewer presynaptic cholinergic neurons surviving to form synaptosomes.

RESULTS

The following table summarises the results of the three assays:

	CONTROL	LESIONED
ChAT ACTIVITY pmol/min/mg protein	334 ± 18 (100%)	280 ± 11 (84%)*
[^{3}H]NICOTINE BINDING fmol/mg protein	23.2 ± 5.2	26.8 ± 4.7
[^{3}H]Ch UPTAKE pmol/mg protein/30 min	72.1 ± 2.7	69.6 ± 9.8

TABLE A.3: EFFECT OF QUISQUALATE LESIONING OF THE MSA ON HIPPOCAMPAL CHOLINERGIC PARAMETERS. Values are mean ± SEM (% of control), n=4. * = significantly different from control at $p<0.05$.

There was a small but significant depletion of ChAT activity in the lesioned group, hence the lesioning procedure was deemed to have been successful. There was no significant reduction in the binding of [^{3}H]nicotine, nor was there a significant reduction in the [^{3}H]Ch uptake of the synaptosomes produced from the lesioned rats compared to the control rats.

DISCUSSION

The results duplicate the finding of the previous lesion study, namely that a deficit in ChAT activity does not necessarily parallel a corresponding decrease in [^{3}H]nicotine binding. The possible reasons for this have been discussed. Interestingly, there was also no decrease in synaptosomal viability as measured by uptake of [^{3}H]Ch, following a 16% reduction in ChAT activity, and this is harder to explain. This contradicts a report by

MacIntosh & Collier (1976) who noted a reduction in high-affinity Ch uptake after septal-hippocampal lesions. If high-affinity uptake of Ch was maintained, there is every reason to believe that large numbers or presynaptic nAChRs could also be intact, obviating the need to invoke postsynaptic nAChR compensation for the apparent lack of change in [^{3}H]nicotine binding sites.

Reports of reduced ChAT activity in Alzheimer patients are numerous (reviewed by Bartus *et al.*, 1982; Rossor, 1982), in fact it was the reports of reduced cortical ChAT activity in post-mortem Alzheimer patients that provided an impetus for the suggestion that the widespread changes seen in end-stage cases of Alzheimer's disease are the result of an underlying neuronal degeneration. The size of the ChAT reduction is dependent upon the brain region tested, selected examples are given below.

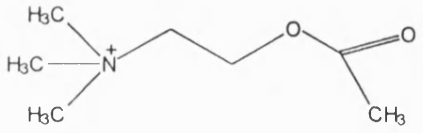
ChAT REDUCTION	BRAIN REGION	STUDY
55%, 65%	MSA; nBM	Henke & Lang, 1983
60-80%	hippocampus	Nordberg *et al.*, 1983
60-70%	various cortical regions	Rossor, 1982
75-80%	hippocampus	Perry *et al.*, 1986; 1987

Generally there is a substantial reduction in ChAT, which the present lesion studies do not model. If a more substantial ChAT reduction had been produced then it would have been reasonable to expect to measure detectable changes in [^{3}H]nicotine binding density. Such changes have been documented in samples from post-mortem Alzheimer patients. For example, Perry *et al.*, (1986), reported an approximate 50% reduction in M2 mAChR binding and nicotinic binding sites in human hippocampus, which is to be expected if there has been extensive degeneration of the septo-hippocampal cholinergic pathway.

In summary, despite measuring significant ChAT deficits in rat hippocampus and cortex, produced by excitotoxic lesions of the MSA and nBM respectively, there were no apparent changes in [^{3}H]nicotine binding density in either region, nor was there a reduction of [^{3}H]Ch uptake into synaptosomes prepared from the hippocampi of lesioned rats compared to controls. The magnitude of the ChAT depletion (approximately 20%) was small and since only a proportion of hippocampal and cortical [^{3}H]nicotine binding sites were thought to be located on presynaptic terminals of cholinergic nerves and hence affected by the lesions, it is feasible that 20% reductions in cholinergic-neuron-associated [^{3}H]nicotine binding density would not be measurable. To represent the cholinergic deficits

seen in post-mortem Alzheimer brains, a lesion producing 70-80% ChAT reductions would be a more accurate model: changes in [^{3}H]nicotine binding density following such a lesion would undoubtedly be more helpful in estimating the relative proportions of presynaptic cholinergic nAChRs.

APPENDIX: CHEMICAL STRUCTURES



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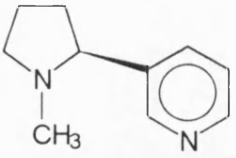

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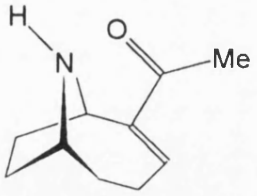
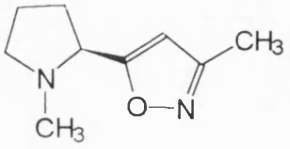


(+) -Anatoxin-a



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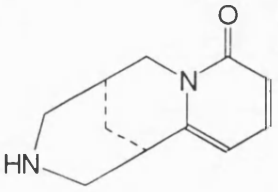

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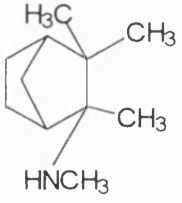
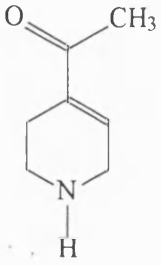
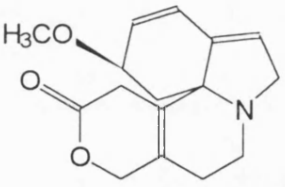


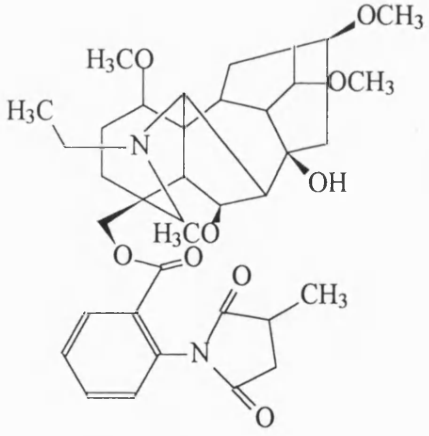
Mecamylamine



Isoarecolone



Dihydroβerythroidine



Methyllycaconitine

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